Stoichiometry of δ subunit containing GABA\(\text{A}\) receptors

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BACKGROUND AND PURPOSE
Although the stoichiometry of the major synaptic αβγ subunit-containing GABA\(\text{A}\) receptors has consensus support for \(2\alpha:2\beta:1\gamma\), a clear view of the stoichiometry of extrasynaptic receptors containing δ subunits has remained elusive. Here we examine the subunit stoichiometry of recombinant α4β3δ receptors using a reporter mutation and a functional electrophysiological approach.

EXPERIMENTAL APPROACH
Using site-directed mutagenesis, we inserted a highly characterized 9′ serine to leucine mutation into the second transmembrane (M2) region of α4, β3 and δ subunits that increases receptor sensitivity to GABA. Whole-cell, GABA-activated currents were recorded from HEK-293 cells co-expressing different combinations of wild-type (WT) and/or mutant α4(L297S), β3(L284S) and δ(L288S) subunits.

KEY RESULTS
Recombinant receptors containing one or more mutant subunits showed increased GABA sensitivity relative to WT receptors by approximately fourfold, independent of the subunit class (α, β or δ) carrying the mutation. GABA dose–response curves of cells co-expressing WT subunits with their respective L9′S mutants exhibited multiple components, with the number of discernible components enabling a subunit stoichiometry of \(2\alpha, 2\beta\) and \(1\delta\) to be deduced for α4β3δ receptors. Varying the cDNA transfection ratio by 10-fold had no significant effect on the number of incorporated δ subunits.

CONCLUSIONS AND IMPLICATIONS
Subunit stoichiometry is an important determinant of GABA\(\text{A}\) receptor function and pharmacology, and δ subunit-containing receptors are important mediators of tonic inhibition in several brain regions. Here we demonstrate a preferred subunit stoichiometry for α4β3δ receptors of \(2\alpha, 2\beta\) and \(1\delta\).

Abbreviations
M2, second transmembrane region; nAChRs, nicotinic acetylcholine receptors; PTX, picrotoxin; SA, spontaneous channel activity; WT, wild-type

Introduction
GABA\(\text{A}\) receptors are the main class of inhibitory ligand-gated ion channels in the mammalian CNS (receptor nomenclature follows Alexander et al., 2013). They are hetero-pentameric complexes forming a central anion-conducting channel. To date, eight classes of GABA\(\text{A}\) receptor subunits have been identified, with half of these exhibiting multiple isoforms: α(1–6), β(1–3), γ(1–3), δ, ε, θ, π and ρ(1–3). Although GABA\(\text{A}\) receptors exhibit distinct regional and developmental expression patterns in the CNS, γ2-containing receptors are considered the dominant subtype found at GABAergic inhibitory
synapses (Somogyi et al., 1996). By contrast, δ-containing receptors are thought to be exclusively found at extrasynaptic sites, where they play an important role in mediating tonic inhibition (Farrant and Nusser, 2005). Extrasynaptic α4βδ receptors have been identified in several neuronal cell types, including dentate gyrus granule cells and thalamic relay neurons (Sur et al., 1999; Peng et al., 2002). In addition, extrasynaptic α6δ and α18 pairs have been identified in cerebellar granule cells (Jones et al., 1997) and hippocampal interneurons (Glykys et al., 2007), respectively, together with extrasynaptic α5βδ and α1β assemblies identified in the hippocampus (Mortensen and Smart, 2006; Glykys et al., 2008).

The subunit composition of GABA_A receptors is an important determinant of their functional properties as demonstrated by the type of α subunit and presence of the γ2 subunit affecting, for example, receptor sensitivity to benzodiazepines (Korpi et al., 2002). Given that orthosteric and many allosteric binding sites on GABA_A receptors are inter- (Sieghart et al., 2012), it becomes important to understand whether there are preferred subunit stoichiometries that will critically define the nature of these subunit interfaces and thus the receptor’s response to ligand binding. Compared with αβγ receptors, we know least about δ subunit-containing GABA_A receptors, which play an important role in mediating tonic inhibition in several brain regions (Brickley et al., 2001; Porcellon et al., 2003; Farrant and Nusser, 2005; Santhakumar et al., 2010). To address this deficit, we have employed a pharmacological analysis, in combination with a reporter mutation, to better understand the structural properties of δ-containing receptors using heterologous expression systems (e.g. HEK-293 and Xenopus laevis oocytes). Previous reports note that some functional discrepancies have been observed for αδβ receptors, such as EC50 values for GABA and ethanol sensitivity (Wallner et al., 2006; Wagoner and Czajkowski, 2010). Although the stoichiometry of major synaptic αβδ GABA_A receptor isoforms has broad consensus support for α1β1γ2δ (Backus et al., 1993; Chang et al., 1996; Tretter et al., 1997), an unequivocal view of the stoichiometry for extrasynaptic δ-containing receptors remains elusive. Although atomic force microscopy of recombinant α4β3δ receptors has suggested a stoichiometry of 2α:2β:1γ (Barrera et al., 2008), biochemical analysis of recombinant α4β2δ receptors indicates that more than one δ can be incorporated into the receptor complex (Wagoner and Czajkowski, 2010). Moreover, it was recently demonstrated on the basis of using α1β3δ (Kaur et al., 2009) and α6β3δ concatemers (Baur et al., 2009) that more than one δ subunit can be incorporated into functional channels, although for the former subtype, a constrained conformation of 2α:2β:1δ most closely resembled the pharmacological profile of unconstrained recombinant α1β3δ receptors (Kaur et al., 2009).

In this study, we have examined the subunit stoichiometry of functional recombinant α4β3δ receptors, utilizing polar substitutions of a highly conserved leucine residue within the second transmembrane region (M2) of GABA_A receptors. This residue exchange acts as a reporter mutation causing a profound increase in agonist potency consequently displacing the agonist dose–response curve (Chang et al., 1996; Chang and Weiss, 1999), as also observed for nicotinic ACh receptors (nAChRs) (Filatov and White, 1995; Labarca et al., 1995) and 5-HT3 receptors (Yakel et al., 1993). The extent of the curve shift is correlated with the number of polar substitutions per ion channel complex, and this has been used to deduce the subunit stoichiometry of recombinant α1β2γ2 GABA_A receptors (Chang et al., 1996). By inserting this highly characterized 9′ serine to leucine (L9S) mutation into α4, β3 and δ subunits, we derive a subunit stoichiometry of 2α:2β:1δ for functional α4β3δ GABA_A receptors. Furthermore, our data indicate that for three different, but commonly used, cDNA transfection ratios, the number of incorporated δ subunits seemingly remains fixed at one.

**Methods**

**Site-directed mutagenesis**

Inverse PCR was used to introduce the M2 leucine-to-serine substitutions into murine α4 and β3, and rat δ subunits. The δ subunit was tagged at the N-terminus (between residues 13 and 14 of the mature protein) with a super ecliptic phluorin. The mutagenic oligonucleotides used to make α4(L297S), β3(L284S) and δ(L288S) were 5′-CAGATGACACCTAAGCATC-3′ (sense), 5′-CCACATGCAACATCAACACTCC-3′ (sense) and 5′-CGACATGACACACTCATGTTA-3′ (sense) respectively. Successful mutations were verified by full DNA sequencing.

**Transient receptor expression in HEK-293 cells**

HEK-293 cells were cultured in DMEM supplemented with 10% v/v fetal calf serum, 2 mM glutamine, 100 U·mL⁻¹ penicillin G and 100 μg·mL⁻¹ streptomycin, and were incubated at 37°C in humidified 95% air and 5% CO2. HEK cells were plated on poly-L-lysine-coated coverslips and transfected using a calcium phosphate protocol. Briefly, cDNA encoding wild-type (WT) and/or mutant α4, β3 and δ subunits, in transfection ratios of 1:1, 1:10 or 10:1:10, were mixed with 340 mM CaCl₂ and an equal volume of HBSS (50 mM HEPES, 280 mM NaCl and 2.8 mM Na₂HPO₄, pH 7.2) to form a precipitate. The total amount of cDNA used for each transfection was 4 μg. The DNA-calcium phosphate suspension was applied to HEK cells, which were incubated overnight, and used for electrophysiology 16–24 h after transfection. For the co-expression experiments, WT subunits and their respective L9S mutants were co-expressed in equal amounts, with an overall transfection ratio remaining at 10α:1β:10δ.

**Electrophysiological recordings**

Transfected HEK cells were placed in a recording chamber and viewed with a Nikon Diaphot microscope (Nikon, Kingston-upon-Thames, UK) and phase contrast optics. Cells were continuously perfused with Krebs solution containing (in mM) 140.0 NaCl, 4.7 KCl, 1.2 MgCl₂, 2.52 CaCl₂, 11.0 glucose and 5.0 HEPES, adjusted to pH 7.4 with 1 M NaOH. Patch pipettes were fire polished to 2–4 MΩ and filled with an intracellular solution containing (in mM) 120 KCl, 1 M CaCl₂, 11 EGTA, 10 HEPES, 1 CaCl₂ and 2 ATP, adjusted to pH 7.2 with 1 M NaOH. Whole-cell agonist-activated currents were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale,
CA, USA). Cells were voltage clamped between −20 and −60 mV, depending on peak current size. Whole-cell currents were filtered at 5 kHz (−36 dB), digitized at 50 kHz via a Digidata 1332A (Molecular Devices) and recorded to disk (Dell Pentium Dual Core Optiplex 960; Dell, Berks, UK). Series resistances were monitored throughout each experiment and deviations >20% resulted in the data being excluded from further analysis.

Data analysis

Currents (I<sub>GABA</sub>) activated by concentrations of GABA (|A|) were normalized to the maximal current caused by a saturating concentration of GABA (I<sub>Max,GABA</sub>). The normalized concentration–response curves were fitted with the following equation using a non-linear least-squares method:

\[
I_{GABA} / I_{Max,GABA} = \sum_{i=1}^{n} \frac{1}{1 + (EC_{50}/|A|)^{I}}
\]

where EC<sub>50</sub> is the GABA concentration inducing a half-maximal current, n is the n<sub>fit</sub>, i is the number of components where j = 1 – 3.

For WT αβδ and α4β3δ receptors, the level of inhibition exhibited by 1 μM Zn<sup>2+</sup> on GABA EC<sub>50</sub> responses for each receptor subtype was also assessed.

The spontaneous channel activity exhibited by each mutant was calculated by expressing the outward current induced by the blocker picrotoxin (I<sub>PX</sub>; 1 mM) as a percentage of the maximum current, defined as the sum of I<sub>Max,GABA</sub> and I<sub>PX</sub>. No spontaneous activity (SA) was observed for WT α4β3δ receptors. The level of SA was quantified according to:

\[
SA(%) = \frac{I_{PXi}}{I_{Pxi} + I_{Max,GABA}}
\]

All data are expressed as mean ± SEM. Where appropriate, statistical analyses were performed using an unpaired Student’s t-test or a one-way ANOVA.

Materials

GABA (Sigma, Dorset, UK) and zinc chloride (BDH Biochemical, Poole, UK) solutions were prepared from 1 M stocks (in water), whereas 1 mM picrotoxin (Sigma) was dissolved in extracellular Krebs solution containing 0.05% v/v dimethyl sulfoxide (Sigma). Drugs were applied via a U-tube application system (Mortensen and Smart, 2007).

Results

Functional expression of WT and L9’S mutant α4, β3 and δ subunits

The highly conserved 9’ leucine residues in α4, β3 and δ subunits (Figure 1) were mutated to serines, producing α4(L297S), β3(L284S) and δ(L288S). These mutated subunits are referred to as α<sub>m</sub>, β<sub>m</sub> and δ<sub>m</sub>, whereas their WT counterparts are designated as α, β and δ. WT αβδ, and mutant αβδ<sub>m</sub>, α<sub>m</sub>βδ and α<sub>m</sub>βδ<sub>m</sub>-expressing cells (Figure 2A), demonstrated a dose-dependent sensitivity to GABA (Figure 2A). Notably, GABA whole-cell currents of mutant subunit-expressing cells exhibited prolonged deactivation phases compared with those for WT αβδ receptors (Figure 2A).

L9’S mutations in α4, β3 and δ subunits increase GABA sensitivity

With regard to channel gating αβδ<sub>m</sub>, α<sub>m</sub>βδ and α<sub>m</sub>δ<sub>m</sub>, but not αβδ receptors, exhibited spontaneous activation in the absence of exogenously applied GABA, which was blocked by the chloride channel blocker, picrotoxin (1 mM; Figure 2B). Expressed as a proportion of the total GABA-activated plus spontaneous current [I<sub>PX</sub>(I<sub>PX</sub> + I<sub>Max,GABA</sub>) Figure 2B inset], the levels of spontaneous receptor activation for αβδ and αβδ<sub>m</sub> receptors were 21.9 ± 5.3 and 16 ± 1% respectively. The highest level of spontaneous activation was exhibited by αβ<sub>m</sub>δ-expressing cells (76.6 ± 6.5%) relative to αβδ<sub>m</sub> (P < 0.01) and αβδ<sub>m</sub> (P < 0.05) receptors (non-parametric ANOVA – Kruskal–Wallis test). The increased degree of spontaneous receptor activation observed for the β mutant is likely to reflect the predominant role this subunit plays in stabilizing open-shut GABA channel conformation(s). It is also noteworthy that β homomers can form spontaneously opening ion channels (Krishek et al., 1996; Davies et al., 1997; Wooltorton et al., 1997; Cestari et al., 2000) unlike their α, γ or δ subunit counterparts.

Both the SA and distinctive deactivation profiles exhibited by mutant subunit-expressing cells confirmed that each mutant was efficiently co-assembled into functional αβδ receptors.

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Figure 1

Primary sequence alignment of the second transmembrane region (M2) of α4, β3 and δ subunits. Prime notation (red) denotes the amino acids comprising the ion channel pore. The conserved hydrophobic 9’ leucine residues are indicated and boxed for α4, β3 and δ subunits, with their numbering in the mature subunit proteins.

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Both the SA and distinctive deactivation profiles exhibited by mutant subunit-expressing cells confirmed that each mutant was efficiently co-assembled into functional αβδ receptors.
The \( \alpha \beta \delta \) receptors exhibited an approximately fourfold (4.15) increase in GABA sensitivity, relative to \( \alpha \beta \delta \) receptors. The increased sensitivity of \( \alpha \beta \delta \) was not attributable to a large population of \( \alpha \beta \) receptors being expressed as the EC\(_{50}\) for \( \alpha \beta \) receptors (1.01 \( \pm \) 0.13 \( \mu \)M; data not shown) is similar to that for \( \alpha \beta \delta \) receptors (1.91 \( \pm \) 0.47 \( \mu \)M; Welch's t-test: \( P = 0.13 \)). By comparison, the GABA sensitivities of \( \alpha \beta \delta \) and \( \alpha \beta \delta \) receptors were higher compared with \( \delta \) receptors, causing shifts of 16 (15.91) and 17 (17.36)-fold, respectively, in the GABA EC\(_{50}\) (Figure 3).

If we assume each subunit mutation has an equivalent effect on GABA potency, independent of the subunit class in which the L9' mutation is inserted, and if we further assume that the receptor contains two \( \alpha \) subunits, then we would expect the shift in EC\(_{50}\) by the 9' mutation to be approximately the square of the change due to a single \( \alpha \) subunit. Thus a shift of 15.91 predicts a single \( \alpha \) subunit would cause a 3.99-fold change in EC\(_{50}\). Similarly for the \( \beta \) subunit, a 17.36-fold shift indicates each \( \beta \) subunit (if two copies are present in the receptor) should cause a 4.17-fold change.

Increasing GABA sensitivity with the number of co-assembled L9'S mutant subunits

For heteromeric muscle nAChRs, each 9' polar substitution within the ion channel confers an additional \(-10\)-fold
Table 1
GABA concentration–response curve parameters for WT and L9S mutant containing αβδ receptors

| Subunit combination | GABA EC50 (μM) | nH | No. of mutants* |
|---------------------|----------------|----|----------------|
| αβδ                 | 1.91 ± 0.47    | 1.06 ± 0.04 | 0              |
| αβδm                | 0.46 ± 0.11    | 1.41 ± 0.12 | 1              |
| αmβδ                | 0.11 ± 0.04    | 0.63 ± 0.06 | 2              |
| αmβδm               | 0.12 ± 0.03    | 1.18 ± 0.19 | 2              |
| αmβδm               | 0.08 ± 0.02    | 0.95 ± 0.07 | 3              |

*Number of mutants within the pentamer assuming a 2α:2β:1δ stoichiometry. GABA dose–response curves were obtained from five to nine HEK cells expressing αβδ, αβδm, αmβδ, αmβδ or αmβδm receptors. The Hill equation was fitted to each data set, and the mean values for GABA potency (EC50) and the Hill slope (nH) are shown in the table as mean ± SEM.

increase in agonist sensitivity (Filatov and White, 1995; Labarca et al., 1995). However, such a linear relationship has not been observed for recombinant α1β2γ2S GABA receptors (Chang and Weiss, 1999), where mutations in α, β and γ subunits contributed unequally to the increased GABA sensitivity, thus prohibiting an estimate of receptor stoichiometry (Chang and Weiss, 1999). By contrast, for our α, β and δ subunit receptors, the shifts appeared more consistent, with each mutation contributing an approximately fourfold increase in GABA sensitivity. We therefore investigated the relationship between GABA potency and the number of mutant substitutions in δ-containing GABA receptors with αmβδm-expressing cells.

Based on our predictions, αmβδm receptors would be expected to contain three mutant subunits (i.e. two α and one δ), and thus exhibit even greater sensitivity to GABA, than either αmβδ (double mutant) or αβδm (single mutant) receptors.

For αmβδm receptors an EC50 of 0.08 ± 0.02 μM (Table 1) was determined, which equates to a 23.87-fold increase in GABA sensitivity, which is greater than that for αmβδ and αmβδ receptors and approximates to a threefold shift (2.88) per mutant subunit. Based on the double mutant receptors, we predicted an approximately fourfold shift per subunit, and thus for three mutant subunits, we might have expected a 64-fold increase in GABA sensitivity. The discrepancy between the predicted and actual shift observed for αmβδm could arise from the δm subunit being absent from αmβδm receptor-expressing cells, leaving cell surface receptors mainly composed of αβ receptors containing just two mutant αm subunits. However, this seemed unlikely given that for αmβδm-expressing cells, there was clear evidence of cell surface δm–GFP fluorescence. Furthermore, αmβδm-expressing cells exhibited a level of SA (49.4 ± 8.4%) that was comparable with the combined spontaneous activities of αmβδ (21.9 ± 5.3%) and αβδm receptors (15.7 ± 1.3%; Figure 2B). Taken together, these data suggest that both αm and δm subunits were efficiently incorporated into functional αmβδm receptors.

Our predicted shift of 64-fold for αmβδm receptors was predicated on the assumption that each additional mutant subunit within the receptor complex acts independently. Although this holds for receptors with two mutant subunits, it is conceivable that with three such substitutions, some degree of interaction between adjacent mutant subunits might cause deviations from the predicted curve shifts.

**cDNA transfection ratio has no effect on α4β3δ receptor stoichiometry**

Recently, using an imaging approach based on inserting bungarotoxin binding sites into GABA receptors, it was demonstrated that the number of δ subunits incorporated into the α4β2δ receptor complex could vary with the cDNA transfection ratio (Wagoner and Czajkowski, 2010). We therefore investigated this possibility using our functional approach with HEK cells transfected with one of the following three commonly used α : β : δ cDNA ratios – 1:1:1, 1:1:10 or 10:1:10 (Borghese et al., 2006; Stórustovu and Ebert, 2006; Barrera et al., 2008; Hoestgaard-Jensen et al., 2010).

First, we ascertained the sensitivity of αβδ-expressing cells to the GABA1 receptor subtype–selective blocker Zn2+ (Smart et al., 1991; Nagaya and Macdonald, 2001; Hosie et al., 2003) to assess if binary αβ constructs were present as 1 μM Zn2+ selectively inhibits αβ to a far greater extent than αβδ receptors (Krishek et al., 1998; Hosie et al., 2003; Stórustovu and Ebert, 2006). Accordingly, 1 μM Zn2+ inhibited the GABA EC50 response of αβδ receptors by 75.5 ± 5.7% (Figure 4A). By contrast, the Zn2+ sensitivity of αβδ-expressing cells did not vary significantly with the αδ transfection ratio (13.1 ± 3.4, 17.0 ± 3.9 and 17.6 ± 0.7%; one-way ANOVA – Bonferroni: P = 0.5), but all were significantly reduced compared with Zn2+ inhibition of αβ δ receptors (Figure 4A; one-way ANOVA – Dunnett’s: P < 0.0001). These data also confirmed the likelihood of efficient incorporation of δ subunits into functional αβδ receptors, for all three transfection ratios used.

We next studied the effect of varying the transfection ratio on GABA sensitivities of αβδ and αβδm receptors. For WT αβδ receptors, altering the transfection ratio had no effect on GABA sensitivity (1.38 ± 0.20, 1.88 ± 0.32 and 1.91 ± 0.47 μM for αβδ receptors of 1:1:1, 1:1:10 and 10:1:10, respectively; one-way ANOVA – Bonferroni: P = 0.56). Similarly, the GABA dose–response curves for αβδm-expressing cells transfected with different ratios were also indistinguishable (Figure 4B), and their GABA EC50 values (0.23 ± 0.01, 0.31 ± 0.08 and 0.46 ± 0.11 μM for αβδm ratios of 1:1:1, 1:1:10 and 10:1:10, respectively) did not differ significantly (one-way ANOVA – Bonferroni: P = 0.20). Although there appears to be a trend for αβδm-expressing cells transfected with a 10:1:10 ratio to have higher nH (1.4 ± 0.2; Figure 4B) than those transfected with either a 1:1:1 (0.82 ± 0.03) or 1:1:10 (0.9 ± 0.2) transfection ratio, this was not significant (Welch’s t-test: P = 0.09 and P = 0.17 respectively).

Overall, altering the transfection ratio had no significant effect on the relative GABA EC50 shifts between δ- and δm-expressing cells (Figure 4B), suggesting that at least for these three transfection ratios, the number of δ subunits incorporated into α4β3δ receptors remains relatively constant.
relative EC$_{50}$ shifts induced by different classes of mutant subunits, but instead relied on co-expressing WT and L9'S mutants to generate multiple populations of receptors expressed in the same cells (Chang et al., 1996).

In principle, the co-expression of WT subunits with their respective L9'S mutants (e.g. α and α$_m$) should introduce discrete and discernible components into the GABA dose–response curve of expressing cells. For example, assuming there are two α subunits per receptor pentamer, these components would represent distinct GABA$_A$ receptors of ααβδ, α$_m$αβδ and ααβδ$'$ and its equivalent, α$_m$αβδ$'$. Thus, the GABA sensitivity that is attributable to an individual receptor population would be observed by an inflection in the dose–response curve. This analysis was used to infer the subunit stoichiometry for αβ2γ2S GABA$_A$ receptors (Chang et al., 1996).

Taking a similar approach for αβ3δ receptors, we generated GABA dose–response curves for cells co-transfected with β, δ and equal amounts of α and α$_m$ cDNAs. The α$_m$αβδ GABA dose–response curve exhibited three discernible components, which were described by the sum of three Hill equations (Figure 5A). The first and third components accounted for 34.0 ± 3.3 and 17.5 ± 2.7% of the total receptor population, with GABA EC$_{50}$ values of 0.023 ± 0.005 and 5.72 ± 0.90 μM respectively (Table 2). These EC$_{50}$ values are in close proximity to those for α$_m$βδ (0.46 ± 0.11 μM) and αβδ (1.9 ± 0.47 μM) receptors, suggesting these two components to the dose–response curves of α$_m$αβδ (Figure 5A) are attributable to α$_m$βδ and αβδ receptors. Moreover, the appearance of an intermediary component with an EC$_{50}$ of 0.30 ± 0.03 μM (48.7 ± 5.6%) suggested the expression of receptor pentamers containing one WT and one mutant subunit (i.e. α$_m$αβδ or α$_m$αβδ$'$. Similar to α$_m$αβδ-expressing cells, the GABA dose–response curves of αβδ$'$-expressing cells revealed three distinct components (Figure 5B). The first component (24.7 ± 3.5%) had an EC$_{50}$ of 0.03 ± 0.01 μM, corresponding to the αβδ$'$ receptor population. Approximately 31.5 ± 2.6% of receptors exhibited an EC$_{50}$ of 4.68 ± 0.74 μM attributable to αβδ receptors. Again, the appearance of an intermediary component with an EC$_{50}$ of 0.31 ± 0.05 μM (43.9 ± 3.5%) was indicative of a third receptor population containing both β subtypes (i.e. β and β$_m$).

By contrast, the mean GABA dose–response curve for αβδ$'$$_m$ receptors did not exhibit obvious multiple components (Figure 5C). However, detailed analysis of individual dose–response curves revealed that for most cells sampled (7/10), two components were discerned (Figure 5C inset). For those cells exhibiting two components, the majority of receptors (75.8 ± 3.0%) exhibited a GABA EC$_{50}$ of 0.21 ± 0.01 μM, whereas 24.2 ± 2.8% of receptors exhibited an EC$_{50}$ of 2.14 ± 0.48 μM. The GABA sensitivities of these two components are similar to the observed EC$_{50}$ for αβδ$'$$_m$ and αβδ receptors respectively (Table 2). Although the remaining cells (3/10) did not overtly display multiple components, their GABA sensitivities were intermediary to those of αβδ$'$ and αβδ$'$$_m$-expressing cells. The absence of a third component suggested that αβδ$'$$_m$-expressing cells exhibit only two receptor populations, αβδ and αβδ$'$$_m$, and thus it follows that each receptor complex is most likely to contain only one δ subunit.

Collectively, these data demonstrate that αβδ receptors are most likely to contain two αs, two βs and one δ subunit.

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Co-expressing WT and mutant subunits confirms αβ3δ receptor stoichiometry

Our deductions so far are based on the assumption that each subunit mutation has an equivalent effect on GABA potency, regardless of the subunit in which the L9’S mutation is inserted. Although this holds for our αβ3δ receptors, some deviation may occur when the number of mutant subunits per receptor is increased, as noted for αβ2γ2S GABA$_A$ receptors (Chang et al., 1996) and heteromeric nAChRs (Labarca et al., 1995). To overcome this methodological limitation, Chang et al. (1996) proposed an alternative approach for deducing subunit stoichiometry that does not rely on the
Discussion

Although the stoichiometry of synaptic α1β2γ2 subunit-containing GABA_A receptors has consensus support for 2α:2β:1γ (Backus et al., 1993; Chang et al., 1996; Tretter et al., 1997), the stoichiometry of extrasynaptic δ-containing receptors remains unclear and potentially variable with a dependence on experimental conditions. This may reflect a difference in the co-assembly properties of δ, with different α and β subunits (Baur et al., 2009; Kaur et al., 2009; Wagoner and Czajkowski, 2010). We used an alternative approach to probe α4β3δ stoichiometry by introducing a well-characterized 9’ leucine-to-serine mutation into the M2 domains of α4, β3 and δ subunits. Each polar substitution increased the GABA sensitivity of mutant subunit-containing receptors (by approximately fourfold) in relative proportion with the number of mutant subunits assembled in the receptor. This, in conjunction with data derived from cells co-expressing mutant and respective WT subunits, revealed a relatively consistent subunit stoichiometry, by these methods, of 2α, 2β and 1δ.

Assumptions and limitations

Our deductions regarding α4β3δ GABA_A receptor stoichiometry are predicated on the assumption that the L9’S mutations do not perturb the ‘normal’ subunit stoichiometry of these receptors. Because N-terminal motifs have been established as the key determinants of GABA_A receptor subunit assembly (Connolly et al., 1996; Taylor et al., 1999; Klausberger et al., 2001), it seemed unlikely that a point mutation within the ion channel-lining M2 region would alter receptor subunit stoichiometry. However, it is intriguing that for most αδβδm-expressing cells, the component that was attributable to αδβδm receptors was larger than that for αδβ receptors (∼75 and 24%, respectively), suggesting that δm might be more efficiently incorporated into functional receptors than δ.

Given the M2 location of the point mutation, a more likely explanation for the disproportionate percentage components is that the mutation may affect the gating kinetics of the receptor. For nAChRs (Filatov and White, 1995) and GABA_A α1β3γ2L receptors (Bianchi and Macdonald, 2001), it has been demonstrated that 9’ mutant-containing receptors can exhibit altered single-channel conductances and/or open probabilities. This could cause the apparent percentage components of αδ and αδm to vary (Chang et al., 1996). Nevertheless, because our conclusions rely on the number of observable components in the dose–response curves and not on the relative contribution of each individual component, our conclusion that α4β3δ receptors contain only one δ subunit still remains valid.

Comparison with previous studies

To date, only two studies have investigated the subunit stoichiometry of unconstrained recombinant α4β2/3δ receptors. Although atomic force microscopy has revealed a subunit stoichiometry of 2α4:2β3:16 (Barrera et al., 2008), the immunopurification of cell surface α4β2δ2 receptors suggested that by increasing the relative abundance of δ, more than one δ can be incorporated into the receptor complex (Wagoner and
Czajkowski, 2010). Moreover, a study using receptor expression in oocytes reported that increasing relative amounts of δ cRNA increased the GABA EC50 and decreased the Hill slopes for α4β3δ GABA dose–response curves (You and Dunn, 2007).

Although a change in stoichiometry may account for altered receptor function, it is also equally plausible, from studies using concatamers and thus constrained subunit positions, that δ subunits may assume various locations within a functional receptor pentamer, and also potentially contribute to an agonist-binding site (Kaur et al., 2009; Sigel et al., 2009). Thus the previously described effects on receptor function may also have arisen from the rearrangement of subunits within the receptor. Indeed, for concatemeric α1β3δ receptors, those with an βδβδ (anticlockwise) subunit arrangement appear to be ~26-fold less sensitive to GABA than receptors with the βδβδ (anticlockwise) subunit arrangement (Kaur et al., 2009), demonstrating the functional importance of subunit location within a receptor pentamer.

Our data indicate that, at least for three commonly used α : β : δ transfection ratios 1:1:1, 1:1:10 or 10:1:10 (Borghese et al., 2006; Stórustovu and Ebert, 2006; Barrera et al., 2008; Hoestgaard-Jensen et al., 2010), the number of incorporated δ subunits seemingly remains fixed at one. Moreover, we found no significant effect of altering cDNA transfection ratio on α4β3δ receptor function. In accordance with our findings, another oocyte study had demonstrated no significant effect of altering cRNA transfection ratio on the sensitivity of WT α4β3δ receptors to GABA or Zn2+ (Borghese and Harris, 2007).

Although the discrepancy between our observations and those previously reported remain unclear, one difference may be the use of different expression systems. Alternatively, the use of different β isoforms may also give rise to these discrepancies. Given that β2 and β3 subunits have been demonstrated to have distinctive assembly properties (Taylor et al., 1999), this might have important implications for their oligomerization with δ subunits.

### Subunit positioning

Although we demonstrate a stoichiometry of 2α:2β:1δ for α4β3δ receptors, our data give little indication of subunit arrangement, which could be an important determinant of αβδ receptor function (Baur et al., 2009; Kaur et al., 2009). The subunit positional arrangement of α1β2 receptors is widely accepted to be βδβδβ (anticlockwise) (Baumann et al., 2001; 2002; Baur et al., 2006; Smart and Paolotti, 2012). Given the conflicting evidence regarding the number of incorporated δ subunits, it is unsurprising that the subunit arrangement of recombinant αβδ remains undefined. For α4β3δ receptors with a stoichiometry of 2α:2β:1δ, structural microscopic analysis has revealed a predominant βδδδ δ anti-clockwise arrangement (Barrera et al., 2008), suggesting δ can assume the position of the γ2 subunit in an αγγγ receptor. However, in the same study, a minority of receptors (~21%) were found to have an alternative βδδδ δ subunit arrangement, indicating more than one arrangement may be possible (Barrera et al., 2008). Indeed it has been recently demonstrated that δ can assume multiple positions when constrained within αβδ concatemers (Baur et al., 2009; Kaur et al., 2009). Intriguingly, concatemeric α4β2δ receptors with the βδδδ conformation (Shu et al., 2012) form functional receptors with similar pharmacological profiles to unconstrained recombinant α4β2δ receptors (Stórustovu and Ebert, 2006), whereas α1β3δ receptors formed from the alternative βδδδ δ anti-clockwise arrangement exhibit similar GABA and Zn2+ sensitivities to non-concatemated receptors (Kaur et al., 2009). These findings suggest that the arrangement of recombinant and native δ-containing receptors is still open to question.

To conclude, we demonstrate that the subunit stoichiometry of heterologously expressed α4β3δ receptors is 2α:2β:1δ. This stoichiometry remains unchanged even with varying cDNA transfection ratios, which may reflect that this is the preferred, dominant subunit assembly for this important extrasynaptic GABA4 receptor subtype that underlies tonic inhibition in some areas of the brain.

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### Conflict of interest

None.
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