Low nanomolar GABA effects at extrasynaptic α4β1/β3δ GABA_A receptor subtypes indicate a different binding mode for GABA at these receptors

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Ionotropic GABA_A receptors are a highly heterogenous population of receptors assembled from a combination of multiple subunits. The aims of this study were to characterize the potency of GABA at human recombinant δ-containing extrasynaptic GABA_A receptors expressed in Xenopus oocytes using the two-electrode voltage clamp technique, and to investigate, using site-directed mutagenesis, the molecular determinants for GABA potency at α4β1δ-containing GABA_A receptors. α4/δ-Containing GABA_A receptors displayed high sensitivity to GABA, with mid-nanomolar concentrations activating α4β1δ (EC50 = 24 nM) and α4β3δ (EC50 = 12 nM) receptors. In the majority of oocytes expressing α4β3δ subtypes, GABA produced a biphasic concentration-response curve, and activated the receptor with low and high concentrations (EC50(1) = 16 nM; EC50(2) = 1.2 μM). At α4β2δ, GABA had low micromolar activity (EC50 = 1 μM). An analysis of 10 N-terminal singly mutated α4β3δ receptors shows that GABA interacts with amino acids different to those reported for α1β2γ2 GABA_A receptors. Residues Y205 and R207 of the β3-subunit significantly affected GABA potency, while the residue F71 of the α4- and the residue Y97 of the β3-subunit did not significantly affect GABA potency. Mutating the residue R218 of the δ-subunit, equivalent to the GABA binding residue R207 of the β2-subunit, reduced the potency of GABA by 670-fold, suggesting a novel GABA binding site at the δ-subunit interface. Taken together, GABA may have different binding modes for extrasynaptic δ-containing GABA_A receptors compared to their synaptic counterparts.

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1. Introduction

γ-Aminobutyric acid (GABA), the principal inhibitory neurotransmitter in the vertebrate central nervous system mediates fast synaptic transmission via ligand-gated GABA_A ion channel receptors. To date, a number of human GABA_A receptor subunit genes have been identified and grouped together by their amino acid sequence similarity. These include α1–6, β1–3, γ1–3, δ, ε, θ, and τ [1–3]. These subunits can mix and match to form possible heteromeric receptor subtypes. Of the six α-subunits, α1–3 are generally localized at synapses and mediate phasic inhibition, while α4–6 dominate sites outside the synapse and mediate tonic inhibition [4]. Receptors containing α4–6 subunits have a higher sensitivity to GABA than their synaptic counterparts [5]. Most GABA_A receptors contain a γ-subunit and these receptors can be found at both synaptic and extrasynaptic locations, whereas the δ-subunit predominates on peri- and extrasynaptic locations [6,7].

The δ-subunit preferentially forms receptors with α4/α6-subunits. The α6/δ-containing receptors are located on cerebellar granule cells, while the α4/δ-containing receptors are present in the dentate gyrus, thalamus, and neostriatum [8]. Recently the δ-subunit was shown to colocalise with the α1-subunit and as such, an α1βδ combination is thought to express at extrasynaptic sites on hippocampal interneurons [9].
Given the distinct location of δ-subunit-containing GABA<sub>A</sub> receptors, their high sensitivity to neurosteroids and lack of sensitivity to benzodiazepines, extraspastic δ-containing receptors have been implicated in stress [10] and disorders associated with the menstrual cycle and puberty [11,12], along with other pathophysiological conditions such as idiopathic generalized and temporal lobe epilepsies [13–16], stroke [17] and fragile X mental retardation [18]. Thus δ-subunit-containing GABA<sub>A</sub> receptors are important targets for drug development [19].

The potency of GABA at GABA<sub>A</sub> receptors is affected by various factors including subunit combination and the presence of endogenous modulators. Given the large number of subunits, there is a high level of structural diversity, yet the impact of this diversity on GABA potency is not known [8,20]. Interestingly, the pharmacology at δ-subunit-containing GABA<sub>A</sub> receptors has become a hotly debated topic as there are inconsistencies between published data [21]; there are conflicting data with regards to the potency of gaboxadol (THIP) [22] and ethanol [21,23], and the promiscuous nature of the δ-subunit makes the stoichiometric composition and subunit arrangement of these receptors difficult to determine [24]. Indeed distinct stoichiometric forms of concatenated αδβδ GABA<sub>A</sub> receptors have been shown to be functional, suggesting a possible GABA binding site at an interface of the δ-subunit [24]. In addition, atomic force microscopy studies showed two distinct subunit arrangements for the α4β3δ GABA<sub>A</sub> receptor [25]. Given the complex issues surrounding the δ-subunit, it is not surprising that there are inconsistencies in the reported data.

It is well established that the GABA binding sites at αβγ GABA<sub>A</sub> receptors are formed by residues of the interfaces between the principal (+) side of the β-subunit and the complementary (−) side of the α-subunit. Specific residues known to form part of the GABA binding site, and interact with the carboxylic acid moiety of GABA to affect its affinity and potency at the αβγ receptor subtype [26–29], are amino acids R207 and Y205 of the β2-subunit, and F64 of the α1-subunit. In addition, residue Y97 of the β2-subunit is known to interact with the amino group of GABA [28,30].

In this study we evaluated the potency of GABA on human recombinant δ-containing receptors expressed in oocytes and provide evidence that GABA can act at low-to-mid-nanomolar concentrations at α4β1/β3 δ GABA<sub>A</sub> receptors, while at α4β2δ GABA<sub>A</sub> receptors, GABA is 100-fold weaker. Investigations into the molecular determinants for the nanomolar GABA effects at α4β3δ GABA<sub>A</sub> receptors revealed that residues, known to interact with GABA at αβγ GABA<sub>A</sub> receptors, are not essential for αδβ activity, and challenges the common assumption that the δ-subunit is merely a γ-substitute.

2. Materials and methods

2.1. Reagents

GABA, HEPES Sodium salt, tricaine, theophylline, pyruvate, zinc chloride and gentamycin were all purchased from Sigma (St. Louis, MO, USA).

2.2. GABA receptor subunit constructs

Human α1 and β2 in pcDM8 was provided by Dr Paul Whiting (Merck, Sharpe and Dohme Research Labs, Harlow, UK), β3 in pGEMHE α4, and δ in pDNA1/Amp and α6 and β1 in pcDM8 were a gift from Dr Bjørke Ebert (H. Lundbeck A/S, Valby, Denmark). cDNA vectors were linearized with the appropriate restriction endonucleases (see Supplementary information Table S1) and capped transcripts were produced from linearized plasmids using the ‘mMessage mMachine’ T7 transcript kit from Ambion (Austin, TX, USA). The quality of mRNA was determined by 0.5% agarose gel electrophoresis. mRNA concentrations were measured by NanoDrop® ND-1000 UV–vis Spectrophotometer. mRNA was diluted with nuclease-free water and stored at –80 °C.

In a second independent study, human α4- and β3-subunits were cloned and sequenced as previously described [31]. The δ-subunit was cloned in-house from human hippocampus poly(A+) mRNA (Clontech, Mountain View, CA, USA), sequenced and matched acc. no NM_000815. mRNA was prepared following the protocol previously described [31].

2.3. Site directed mutagenesis

Site-specific mutations were introduced into the cDNAs of the GABA<sub>A</sub> receptor subunits using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as described previously [32]. Mutant clones were submitted for complete sequencing to confirm the successful incorporation of the point mutation and absence of spurious mutations.

2.4. Expression of recombinant GABA receptors in Xenopus oocytes

The methods for oocytes harvesting and preparation have been described previously [33]. Stages V–VI oocytes were sorted and injected (Nanoject, Drummond Scientific Co., Broomali, PA, USA) with cRNA reconstituted in nuclease free water in a ratio of 1:1 for α1:β2/β3 except 5:1 for α4/α6:β3, 1:1:5 for α4/β1/β2:δ and 5:1:5 for α4/β3:δ receptors. For the expression of mutant receptors, a ratio of 5:1 for α4:β3 and 5:1:5 for α4/β3:δ were injected. A summary of the amounts of total wildtype and or mutant cRNA and ratios can be found in the Supplementary information (Table S2).

Oocytes were incubated for up to 3–8 days in standard ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 5 mM HEPES, pH 7.4), supplemented with pyruvate (5 mM), gentamycin (50 μg/mL) and 2% horse serum at 18 °C.

In the second independent study, the cRNA of the α4-, β3- and δ-subunits was mixed in a 1:1:2 ratio to a final concentration of 0.5 μg/μL. The cRNA was injected into the stages V–VI oocytes obtained from Eckoye (Eckoye Bioscience, Castrop-Rauxel, Germany), and incubated for 5–8 days at 18 °C in Modified Barth solution (90 mM NaCl, 1 mM KCl, 0.66 mM NaNO<sub>3</sub>, 2.4 mM NaHCO<sub>3</sub>, 0.82 mM MgCl<sub>2</sub>, 0.74 mM CaCl<sub>2</sub>, 10 mM Na-HEPES and 0.1 g/L gentamycin, adjusted pH to 7.55 with NaOH and filtered through an 0.22 μm filter).

2.5. Electrophysiology

Currents were recorded using the two-electrode voltage clamp (TEVC) technique as described elsewhere [33]. Oocytes were individually placed in a 100 μl chamber connected to a reservoir bottle containing ND96 solution. Glass microelectrodes were made using a micropipette puller (Narishige Scientific Instrument Laboratory, Tokyo, Japan) and filled with 3 M KCl (0.5–2 MΩ). The oocytes were impaled and the membrane potential was clamped at –60 mV while continuously superfused with ND96 solution. Current amplitudes were calculated off-line using Chart software v3.6 (ADInstruments, NSW, Australia).

In order to monitor the receptor composition, Zn<sup>2+</sup> was used to investigate the incorporation of the δ-subunit as outlined in [34]. Thus all binary and ternary receptor complexes with or without mutations were screened with two concentrations of Zn<sup>2+</sup> (0.1 and 1 μM) against GABA EC<sub>50</sub> for that particular receptor subtype before recording GABA concentration-response curves.

In the second independent study, the currents were recorded using TEVC as described by Mirza et al. [31], and the current
amplitudes were calculated off-line using pClamp 10.2 (Molecular Devices, Sunnyvale, CA).

2.6. Data and statistical analysis

Responses to GABA applications were normalized as \( \% = (I/I_{\text{max}}) \times 100 \), where \( I \) is the peak amplitude of current response and \( I_{\text{max}} \) is the maximal peak current produced by GABA measured in each individual cell. Normalized responses were pooled and graphed as means \( \pm \) SEM from at least three oocytes from at least two different batches. Responses were fitted to the four-parameter logistic equation: \( I = I_{\text{max}}/(1 + [EC_{50}/A]^n) \); alternatively, the data were fitted with a sum of two Hill equations

\[
I = \frac{I_{\text{max}}}{(1 + [EC_{50}/A]^n)} + \frac{I_{\text{max}}^2}{(1 + [EC_{50}/A]^n)}
\]

where \( I \) is the peak amplitude of the current elicited by a given concentration of agonist \( [A] \), \( I_{\text{max}} \) is the maximum amplitude of the current, \( EC_{50} \) is the concentration required for half-maximal response, and \( n_h \) is the Hill slope (Prism 5 GraphPad software, San Diego, CA). An \( F \) test was used to determine which fit is the preferred model.

All statistical calculations are presented as mean \( \pm \) standard error of the mean (SEM) or as mean (95% confidence intervals (CI)). When two groups were compared, the Student’s \( t \)-test was used and when more than two groups were compared, One way ANOVA followed by Tukey’s or Dunnnett’s post hoc tests were used.

3. Results

3.1. Evaluating the potency of GABA on binary \( \alpha \beta \) and ternary \( \alpha \beta \delta \) receptors

In order to establish the potency of GABA at \( \delta \)-containing GABA\(_{A}\) receptors, we evaluated GABA at binary and ternary receptors composed of \( \alpha 1\beta 3 \), \( \alpha 4\alpha 6\beta 1–3 \) and \( \alpha 4\alpha 6\beta 1–3\delta \) subunits. To check for the incorporation of the \( \delta \)-subunit into receptor complexes, the discriminatory inhibitory activity of Zn\(^{2+} \) at \( \alpha \beta \) versus \( \alpha \beta \delta \) receptors was utilized as an indirect measure [5,35,36]. In our hands, 0.1 and 1 \( \mu \)M Zn\(^{2+} \) was able to inhibit the current produced by GABA (10 \( \mu \)M) at \( \alpha 4\beta 3 \) receptors by 60 \( \pm \) 5\% and 90 \( \pm \) 2\% respectively (\( n = 4 \); Fig. 1A) whereas at \( \alpha 4\beta 3\delta \), Zn\(^{2+} \) (1 \( \mu \)M) was able to partially inhibit the response of GABA and only when the GABA concentration was increased to 3 \( \mu \)M, a concentration higher than the reported \( EC_{50} \) at this receptor [5]. Thus Zn\(^{2+} \) (1 \( \mu \)M) inhibited GABA (0.1 \( \mu \)M) by only 10 \( \pm \) 3\% (\( n = 8 \); Fig. 1B) while the same concentration of Zn\(^{2+} \) (1 \( \mu \)M) inhibited GABA (3 \( \mu \)M) by 40 \( \pm \) 5\% (\( n = 8 \); Fig. 1C and D). These data ascertained that ternary \( \delta \)-containing receptors were expressed but also pointed out that a proportion of receptors may be of a binary \( \alpha \beta \) composition. Similar results were obtained with \( \alpha 1\beta 3(\delta) \) receptors (Supplementary information, Fig. S1).

The holding currents for the binary \( \alpha 1\beta 3 \), \( \alpha 4\alpha 6\beta 1–3 \) receptors clamped at \(-60 \) mV ranged between 0 and \(-20 \) nA, indicating that the receptors were not constitutively active. The \( EC_{50} \) values varied between 0.5 and 90 \( \mu \)M (Table 1), with the

![Fig. 1](image-url) Example of oocyte recordings (current (nA) vs time (s)) illustrating the GABA-induced response and the effect of Zn\(^{2+} \). Zn\(^{2+} \) (0.1 and 1 \( \mu \)M) inhibited (A) GABA (10 \( \mu \)M) at \( \alpha 4\beta 3 \) receptors by 55\% and 95\% respectively. (B) GABA (0.1 \( \mu \)M) at \( \alpha 4\beta 3\delta \) receptors by 5\% and 10\% respectively. (C) GABA (3 \( \mu \)M) at \( \alpha 4\beta 3\delta \) receptors by 40\% and 58\% respectively. GABA was applied alone and in the presence of Zn\(^{2+} \) at the concentrations indicated. Horizontal bars represent duration of application. (D) Average percentage inhibition of GABA (3 \( \mu \)M) by Zn\(^{2+} \) (0.1 and 1 \( \mu \)M) at \( \alpha 4\beta 3\delta \) receptors. Zn\(^{2+} \) (1 \( \mu \)M) significantly inhibited GABA (3 \( \mu \)M) by 38 \( \pm \) 3\% (ANOVA followed by Dunnett’s post hoc test; \( n = 8 \); \( * \)p < 0.01 compared to GABA alone).
Table 1
Effect of GABA on GABA<sub>A</sub> receptors containing or not containing the δ-subunit.

<table>
<thead>
<tr>
<th>Receptor subtype</th>
<th>GABA EC&lt;sub&gt;50&lt;/sub&gt; (µM) (95% CI)</th>
<th>n&lt;sub&gt;H&lt;/sub&gt;</th>
<th>Holding current (mean ± SEM) nA</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>α4β3</td>
<td>34.5 (23.4–50.8)</td>
<td>0.8 ± 0.3</td>
<td>100 ± 5</td>
<td>8</td>
</tr>
<tr>
<td>α4β1</td>
<td>0.72 (0.6 ± 0.8)</td>
<td>1.0 ± 0.2</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>α4β2</td>
<td>2.3 (1.5–3.4)</td>
<td>0.9 ± 0.3</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>α4β3</td>
<td>0.52 (0.29–0.97)</td>
<td>0.6 ± 0.2</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>α6β1</td>
<td>7.7 (5.0–11.6)</td>
<td>1.2 ± 0.2</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>α6β2</td>
<td>2 (1.4–2.8)</td>
<td>1.3 ± 0.3</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>α6β3</td>
<td>1.7 (0.8–3.5)</td>
<td>0.5 ± 0.2</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>α1β3δ</td>
<td>8.7 (6.4–11.7)</td>
<td>0.8 ± 0.3</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>α6β1δ&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.35 (0.24–0.52)</td>
<td>0.9 ± 0.3</td>
<td>−110 ± 15</td>
<td>6</td>
</tr>
<tr>
<td>α6β2δ&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(1)±0.05 (0.02–0.15)</td>
<td>(1)±0.9 ± 0.3</td>
<td>−180 ± 30</td>
<td>4</td>
</tr>
<tr>
<td>α6β3δ&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(2)±8.7 (3.7–20)</td>
<td>(2)±1.7 ± 1</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>α6β3δ&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.44 (0.33–0.53)</td>
<td>0.9 ± 0.4</td>
<td>−80 ± 20</td>
<td>10</td>
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<tr>
<td>α4β1δ&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.024 (0.019–0.030)</td>
<td>1.1 ± 0.1</td>
<td>−350 ± 35</td>
<td>8</td>
</tr>
<tr>
<td>α4β2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.00 (0.89–1.31)</td>
<td>1.3 ± 0.2</td>
<td>−60 ± 20</td>
<td>4</td>
</tr>
<tr>
<td>α4β3δ biphatic 5:1:5 injection ratio&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>(1)±0.012 (0.006–0.025)</td>
<td>(1)±1.2 ± 0.5</td>
<td>−675 ± 65</td>
<td>24</td>
</tr>
<tr>
<td>α4β3δ biphatic 1:1:2 injection ratio&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(1)±0.008 (0.004–0.016)</td>
<td>(1)±0.9 ± 0.3</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>α4β3δ monophatic&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>(2)±1.6 (0.5–4.9)</td>
<td>(2)±1.5 ± 1</td>
<td></td>
<td>5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Constitutively active GABA<sub>A</sub> receptor subtypes.

<sup>b</sup> Data obtained at the Faculty of Pharmacy, the University of Sydney Australia.

<sup>c</sup> Data obtained from Neurosearch A/S, Ballerup, Denmark.

α4β3 subtype being the most sensitive to GABA (Fig. 2A; Table 1; EC<sub>50</sub> = 520 nM; n<sub>H</sub> = 0.6 ± 0.2).

In contrast, GABA<sub>A</sub> receptors formed from α4/α6β1δ, α4β2δ and α1/α6β3δ displayed properties of constitutive activity with holding currents ranging from −100 to (−800) nA when clamped at −60 mV (Table 1). Although we cannot state that there is a relationship between constitutive activity and the expression of δ-containing receptors, oocytes displaying constitutive activity were more likely to be expressing the high affinity GABA receptor. GABA further activated these receptors in a concentration dependent manner with the highest potency being displayed for α4β1δ (Table 1; Fig. 2B; EC<sub>50</sub> = 24 nM; 95% CI 19–30 nM) and α4β3δ (EC<sub>50</sub> = 16 nM; 95% CI 14–18 nM) and the lowest potency displayed for the α1β3δ GABA<sub>A</sub> subtype (EC<sub>50</sub> = 8.7 µM; Table 1; p < 0.05; ANOVA followed by Tukey’s post hoc test). The EC<sub>50</sub> of GABA at α4β2δ, α6β1δ and α6β3δ did not significantly differ (p > 0.05; ANOVA followed by Tukey’s post hoc test) and varied between 0.35 and 1.0 µM (Fig. 2B; Table 1). As reported by others [37], GABA activated α6β2δ GABA<sub>A</sub> subtypes in a concentration dependent manner producing a biphasic concentration-response curve (Supplementary information Fig. S3).

3.2. GABA exerts a biphasic response at α4β3δ GABA<sub>A</sub> receptors

In the majority of α4β3δ-expressing oocytes (24 out of 29 oocytes), the concentration-response curve for GABA displayed a shallow Hill slope (n<sub>H</sub> ≤ 0.6) with an EC<sub>50</sub> of 0.4 µM, similar to the EC<sub>50</sub> of GABA at α4β3 receptors (Fig. 3A). The low Hill slope (n<sub>H</sub>) for α4β3δ-expressing oocytes indicated either negative co-operativity or the presence of a mixed population of receptors with different agonist sensitivities. Indeed, when fitting the data, a two-site model was clearly preferred (Fig. 3B versus A; F (DFn, DFd) = 9.028 (3, 150)). Thus, two distinct sensitivities for GABA were identified: one in the low-to-mid-nanomolar and one in the low micromolar range. The mid-nanomolar EC<sub>50</sub> value for GABA at the α4β3δ subtype was 12 nM (95% CI: 6–25 nM), while the low micromolar EC<sub>50</sub> value for GABA was 1.3 µM (95% CI: 0.7–2.5 µM). The latter value was not significantly different to the EC<sub>50</sub> of GABA at binary α4β3 receptors (Student’s t-test; p = 0.07). Given that Zn<sup>2+</sup> can inhibit a high concentration of GABA at α4β3δ receptors, it could be inferred that the biphasic response curve was due to a mixed population of receptors composed of α4δ and α4β3 GABA<sub>A</sub> receptors. Furthermore, injection of α4 and δ mRNA alone and α4 and δ together did not form functional homomeric or heteromeric GABA<sub>A</sub> gated channels (data not shown).

In 5 out of 29 oocytes expressing α4β3δ GABA<sub>A</sub> receptors, the Hill slope was 0.8 ± 0.1 (Table 1; Fig. 3C) and data were preferentially fitted to a one-site model. The EC<sub>50</sub> value for GABA was 16 nM, approximately 32 times lower than the EC<sub>50</sub> value obtained for GABA.
Fig. 3. (A) Concentration–response curve for GABA at α4β3δ receptors (n = 24) fitted with a one-site model. (B) Concentration–response curve for GABA at α4β3δ receptors fitted with a two-site model (n = 24). (C) Concentration–response curve for GABA at α4β3δ receptors fitted with a two-site model (n = 24). Data are expressed as mean ± SEM (n = 24). α4β3δ injected with a 5:1:5 cRNA ratio and fitted to a one-site model (▲, n = 5), α4β3δ injected with a 1:1:2 ratio and fitted to a two-site model (○, n = 8).

at α4β3 receptors and was not significantly different from the low-to-mid-nanomolar EC50 value obtained from the biphasic nature of the receptor. An example of an oocyte expressing a single population of receptors composed of α4β3δ GABA receptors is given in the Supplementary information Fig. S3.

A completely independent set of recordings obtained at Neurosearch (details outlined in Section 2), confirmed our findings in regard to the potency of GABA and the biphasic nature of the concentration–response curve. At Neurosearch, the injection ratio of cRNA for α4, β3, δ was 1:1:2 and different to our 5:1:5 injection ratio. Varying the injection ratio of GABAδ enabled the fraction of expressed receptors. This approach is extensively used for studying the various stoichiometric forms of nicotinic acetylcholine α4β2 receptor subtypes [38]. When a 1:1:2 injection ratio was used, a 70 ± 10% “high” versus 30 ± 10% “low” potency receptor fraction was observed (Fig. 3C). In contrast, when a 5:1:5 injection ratio was used, there was a 45 ± 10% “high” versus a 55 ± 10% “low” potency receptor population. The EC50 values obtained for the 1:1:2 injection ratio (n = 6) were 8 nM (95% CI: 4–16 nM) and 1.6 μM (95% CI: 0.5–4.9 μM), respectively, and were not significantly different to the EC50 values obtained from the 5:1:5 injection ratio (n = 24); p > 0.05; Student’s t-test.

3.3. Molecular determinants for GABA sensitivity at binary α4β3 GABAδ receptors

The dramatic increase in GABA potency at α4β3δ receptors prompted us to examine the importance of the canonical GABA interaction residues for receptor activation, thus examining initially the effects of creating point mutations in α4- and β3- for α4β3 δ GABA activity. Specifically, mutant subunits α4F71L, β3Y205A, β3R207A and β3Y97A were generated which correspond to α1F64 [26], β2Y205, β2R207 [27,29] and β2Y97 [28,30].

The specific binary combinations included, α4β3Y97A, α4β3Y205A, α4β3R207A and α4F71Lβ3 GABAδ receptors. As shown in Fig. 4, GABA activated currents in a concentration-dependent manner at all binary combinations. Examples of a GABA trace for α4F71Lβ3 and α4β3R207A are shown in Fig. 4A and B, respectively. As expected, the potency of GABA decreased at all combinations tested, shifting the concentration–response curve of GABA to the right compared to wildtype α4β3 receptors (Fig. 4A; Table 2). The EC50 values for GABA were 11.5–4410–1040– and 24 times higher at α4β3Y97A, α4β3Y205A, α4β3R207A and α4F71Lβ3 than at wildtype α4β3 receptors, respectively.

3.4. Molecular determinants for GABA sensitivity at ternary α4β3δ GABAδ receptors

All tested mutant ternary receptors were found to be constitutively active (holding currents varying between −140 and −500 nA) and sensitive to GABA stimulation in a concentration-dependent manner, fitting best with a one-site model of activation (Table 2). For all mutants, we used the lack of Zn2+ inhibition as a proof for δ-subunit incorporation (Supplementary information Fig. S4).

At two of the mutated receptors (α4F71Lβ3δ (Fig. 5A) and α4β3Y97Aδ), GABA was only slightly affected, with 5.5- and 11.5-fold weaker potency relative to their wildtype counterparts. In contrast, mutating the β3 residues Y205 and R207 to alanine had a dramatic effect on GABA potency, leading to 39,940- and 12,310-fold reductions, respectively, compared to the wildtype receptor.

Fig. 4. Representative current traces showing the effect of GABA at binary (A) α4F71Lβ3; (B) α4β3R207A mutant GABAδ receptors. Horizontal bars represent the duration of drug application. (C) Concentration–response curves for GABA at wild-type binary combinations for α4β3 (▲), α4β3Y205A (●), α4β3R207A (●), α4F71Lβ3 (□) and α4β3Y97A (○) mutant receptors. Data represent mean ± SEM (n = 4–8).
Table 2

<table>
<thead>
<tr>
<th>Receptor subtypes</th>
<th>EC50 (µM) (95% CI)</th>
<th>nσ</th>
<th>EC50 (mutant)/EC50 (wildtype)</th>
<th>Holding current (mean ± SEM) nA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>α4β3</td>
<td>0.52 (0.29–0.97)</td>
<td>0.6 ± 0.2</td>
<td>–</td>
<td>–</td>
<td>6</td>
</tr>
<tr>
<td>α4β1β3</td>
<td>12.7 (7.9–20.5)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.5 ± 0.1</td>
<td>24</td>
<td>–</td>
<td>4</td>
</tr>
<tr>
<td>α4β3Y97A</td>
<td>6.0 (3.2–11.3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.6 ± 0.1</td>
<td>11.5</td>
<td>–</td>
<td>4</td>
</tr>
<tr>
<td>α4β3Y205A</td>
<td>2293 (1298–4051)&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.7 ± 0.1</td>
<td>4410</td>
<td>–</td>
<td>4</td>
</tr>
<tr>
<td>α4β3R207A</td>
<td>540 (273–1066)&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.6 ± 0.1</td>
<td>1040</td>
<td>–</td>
<td>4</td>
</tr>
<tr>
<td>α4β3S&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.016 (0.014–0.018)</td>
<td>0.8 ± 0.1</td>
<td>–</td>
<td>– 675 ± 65</td>
<td>5</td>
</tr>
<tr>
<td>α4β1β3S&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.08 (0.07–0.10)&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.9 ± 0.3</td>
<td>5</td>
<td>– 220 ± 60</td>
<td>4</td>
</tr>
<tr>
<td>α4β3Y97Aβ&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.17 (0.06–0.32)&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.6 ± 0.1</td>
<td>10</td>
<td>– 260 ± 70</td>
<td>4</td>
</tr>
<tr>
<td>α4β3Y205Aβ&lt;sup&gt;a&lt;/sup&gt;</td>
<td>639 (454–900)</td>
<td>0.8 ± 0.2</td>
<td>39,940</td>
<td>– 410 ± 55</td>
<td>5</td>
</tr>
<tr>
<td>α4β3R207Aβ&lt;sup&gt;a&lt;/sup&gt;</td>
<td>197 (152–256)&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.8 ± 0.3</td>
<td>12,310</td>
<td>– 350 ± 49</td>
<td>4</td>
</tr>
<tr>
<td>α4β3R218A</td>
<td>10.7 (7.1–16.2)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.6 ± 0.1</td>
<td>670</td>
<td>– 235 ± 49</td>
<td>8</td>
</tr>
</tbody>
</table>

<sup>a</sup> High affinity value only.
<sup>b</sup> Holding currents for constitutive active receptor subtypes.
<sup>*</sup> p < 0.05.
<sup>**</sup> p < 0.01.
<sup>***</sup> p < 0.001 using ANOVA followed by Dunnet post hoc test for comparison against wildtype receptors.
<sup>**</sup>Not significant against wildtype receptors.

We examined the homologous residue to β3R207 on the δ-subunit, and evaluated the potency of GABA when the residue was mutated to alanine. At α4β3R218A mutant receptors, the potency of GABA was dramatically reduced (670-fold reduction (p < 0.001; Student’s t-test, n = 8; Fig. 5B and C; Table 2), suggesting that the δR218 residue plays an important role for receptor activity. Interestingly, compared to all other ternary mutants, GABA at this mutant produced macroscopic current responses that were fast inactivating (Fig. 5B), indicating possible effects on the gating mechanism. However, as these experiments were done using whole cell voltage clamp recordings, conclusions on this matter cannot be made.

4. Discussion

In this study we evaluated the effect of GABA at recombinant δ-subunit-containing GABA<sub>A</sub> receptors expressed in oocytes. In addition, we used α4β(δ) receptors to investigate the role of a number of amino acid residues located in the N-terminal domain and known to contribute to GABA binding and potency. Our results and those of others [5,23] show that δ-containing receptors (α1β3, αβ1–3δ, and αβ1–3δ) are highly sensitive to GABA. However from our studies, GABA was 50–100-fold more potent on α4β1/β3 δ GABA<sub>A</sub> receptor subtypes than on α4β2 subtypes and differed in that the potency of GABA at α4β1/β3 δ GABA<sub>A</sub> receptors was 10–100-fold more potent than previously reported [5]. Low GABA concentrations ranging from 0.1 to 0.8 µM [39,40] have been reported to occur at various extrasynaptic sites in the rodent brain [41] that express high-affinity functional receptors, indicating that variability in brain GABA concentrations may reflect the concentrations of GABA required to activate a particular subtype of δ-containing receptor expressed at the various anatomical sites.

A lot of conflicting pharmacological data exist for the α4β3 δ GABA<sub>A</sub> receptor [5,21–23,42], possibly relating to the δ-subunit’s “promiscuous” nature. The ability of the δ-subunit to take the place of either an α- or β-subunit [24,43,44] makes understanding the pharmacology of δ-containing GABA<sub>A</sub> receptors quite difficult and complex. While our study, in one regard, adds to the complexity of information by reporting yet another GABA EC50 value for α4β3 receptors, it also provides some insight into explaining the controversies reported with respect to the pharmacology of these receptors [5,21–23,42].

Our studies show that GABA activates α4β3 δ GABA<sub>A</sub> receptors with “high” and “low” potencies, indicating that more than one receptor population is expressed. This bipolar concentration–response curve to GABA may be explained in two ways: either
different stoichiometric forms or subunit arrangements for α4β3δ GABA<sub>A</sub> receptors are being formed or a mixture of both binary and ternary receptors is present.

Studies using concatenated receptors composed of α1/α6-, β3- and δ-subunits identified five functional receptors with various stoichiometries [24,43,44], while increasing concentrations of δ-subunit cDNA transfected in HEK-293 cells [45] or cRNA injected in oocytes [46] increases the likelihood of δ-subunit expression, potentially producing mixed receptor populations that are functional. In support of the existence of different subunit arrangements, Barrera and colleagues used atomic force microscopy to reveal that α4β3δ GABA<sub>A</sub> receptors expressed on tsa 201 cells form in two different arrangements composed of the counter-clockwise arrangements β-α-β-α-δ and α-β-α-β-δ [25] (Fig. 6). Recently, the concatenated form of α4-β2-α4-β2-δ was reported indicating that this arrangement [and possibly others] is functional [47]. Should different stoichiometric forms or arrangements exist for α4β3δ GABA<sub>A</sub> receptors then GABA may activate these combinations with different potencies because the binding site(s) for GABA are non-equivalent.

The GABA binding site is believed to be located at the interface of a β(+-) and α(-) subunit. The counter-clockwise arrangement β-α-β-α-δ contains two β(+-) and α(-) interfaces while the counter-clockwise arrangement α-β-α-β-δ has only one (Fig. 6). Thus GABA may act with low potency at one arrangement and high potency at the other. Indeed “high” and “low” potency receptors have been reported for α6β2δ receptors with GABA and other agonists exhibiting distinct EC<sub>50</sub> values [37].

Whilst we cannot rule out the possibility that stoichiometric forms of α4β3δ GABA<sub>A</sub> receptors exist, there are several reasons to indicate that the “high” and “low” potency receptors are due to the expression of α4β3δ and ‘contamination’ of α4β3 GABA<sub>A</sub> receptors: Zn<sup>2+</sup> is a potent inhibitor of binary GABA<sub>A</sub> receptors composed of αβ subunits and in our studies Zn<sup>2+</sup> partially inhibited the response of GABA, indicating that a population of the receptors may not contain the δ-subunit. Supporting this claim, Zn<sup>2+</sup> inhibited more of the GABA response from singularly injected mRNA of α4-, β2-, δ-subunits in oocytes, indicating mixed populations of binary and possibly homomeric receptors composed of β2-subunits, than the concatenated form of α4-β2-δ receptors [47]; the potency of GABA at the “low” potency site was not significantly different to the potency of GABA at binary α4β3 GABA<sub>A</sub> receptors; and the fraction of the “high” potency site can vary with the amount of α4- and or δ-subunit mRNA injected in oocytes (from 5:1:5 to 1:1:2). It is interesting to note that robust binary α4β3 receptors were only observed when a 5:1 mRNA injection ratio was used. In addition, Meera and colleagues showed that gaboxadol (THIP) also activates α4β3δ GABA<sub>A</sub> receptors with “high” and “low” potencies [22]. They concluded that only the “high” potency response exhibited by gaboxadol (THIP) was due to ternary α4β3δ and that the “low” potency response was due to binary α4β3 GABA<sub>A</sub> receptors, i.e. the δ-subunit was not fully incorporated.

In an attempt to further understand how GABA activates α4β3 and α4β3δ GABA<sub>A</sub> receptors, we evaluated the effects of certain amino acid residues located in the N-terminal region known to affect GABA potency. The residues R207 and Y205 in β2- and the residue F64 in α1-subunits are all known to form part of the GABA binding site, located at the interface of the principal β(+-) and complementary α(-) subunits of synaptic αγγ γ receptor subtypes [26,27,29]. We found that the potency of GABA at α4β3 and α4β3δ receptors was strongly affected by mutating the corresponding amino acids β3R207 and β3Y205. Surprisingly, the potency of GABA was less dramatically affected than expected when the α4F71 residue was mutated to alanine. There was a 24-fold reduction in the potency of GABA at α4β3 but only a 5-fold reduction at α4β3δ receptors. This indicates that GABA may have a different binding mode at α4β3 and α4β3δ receptors than at α1β2 and α1β2γ2 GABA<sub>A</sub> receptors where the corresponding mutation in the α1-subunit right-shifts the GABA curve by 72-[28] and 210-fold [26], respectively. Thus F71 of the α4-subunit may not significantly contribute to GABA binding at these receptors.

A similar observation was made for the β3Y97A mutant which did not dramatically affect GABA potency at either α4β3 or α4β3δ GABA<sub>A</sub> receptors. This residue is situated on the principal (+) side of the β-subunit and has previously been shown (in β2) to interact with the amino group of GABA via a cation–π interaction [30]. In fact, mutating β3Y97 to alanine only reduced the potency of GABA by 10-fold, implying that this residue may not contribute to the binding of GABA in the same way as it does in α1β2γ2 receptors [28,30].

![Fig. 6. Cartoon illustrating the possible arrangements for α4β3δ as described by Barrera and colleagues [25]. Binding sites for GABA (triangles) are indicated as well as the studied mutations on the principal β(+) or δ(+) sides or in the complementary α(-) sides. In the counter-clockwise arrangement β-α-β-α-δ (A), a GABA binding site may potentially exist at the δ(+) interface in addition to the two β(+) participating interfaces. In the counter-clockwise arrangement α-β-α-β-δ (B), three non-equivalent GABA sites are possible: two at the β(+) interfaces, and one at the δ(+) interface. The “high” and “low” potency effects exerted by GABA may arise from interaction at either of the two arrangements.](image-url)
Interestingly the δ-subunit residue R218, corresponding to β2R207 in sequence alignments (Fig. 6), was found to significantly affect GABA potency. The 670-fold reduction in potency of α4β3δR218A compared to wild-type receptors may indicate a central role in gating or the existence of an alternative GABA binding site for this residue within the α4β3δ complex. Indeed studies using concatenated αδβ3δ receptors indicate that a GABA binding site could exist at the δ-subunit interface as this subunit takes the place of either α- or β-subunit to form functional receptors [24]. This potential binding site would be in addition to the β(+) interface: as a δ(+)βδ(−) interface for the clock-wise arrangement and a δ(+)αδ(−) and/or a β3(+)δ(−) interface for the anticlockwise arrangement.

The fact that mutations in the β3 subunit (Y205 and R207) have much greater effects on GABA potency than the δR218 mutation suggests that GABA binding at the β3(+)αδ(−) interface is also necessary for channel activation, but the putative binding site containing the δ-subunit may act co-operatively in channel activation. Obviously it is also tempting to speculate that the δ-subunit forms an additional binding site responsible for the “high” potency GABA site. However, as the location of the residue (in the so-called loop C region) is also known to move during channel gating, it cannot be ruled out that δR218 is solely a gating-relevant residue [48]. To further explore this possibility, single channel recordings, and further mutational and binding studies are warranted.

In summary, our data show that GABA exhibits mid-nanomolar potency at α4β1β3 δGABA receptors and that differences in potency amongst research groups may, in part, be the result of varying fractions of binary α4β1β3 and ternary α4β1β3δ receptor expression. In addition, mutational analysis shows that the potency of GABA at α4β3δ receptors is dependent on different amino acid residues at homologous sites involving β(+) and δ(+) interfaces domains than those of α1β2 and α1β2γ δGABA receptors. Our findings raise the possibility that the δ-subunit contributes to an alternative GABA binding site and thus hold interesting implications for further discerning the molecular pharmacology and potential clinical value of the large variety of δGABA receptor subtypes.

Conflicts of interest statement

The authors have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bcp.2012.05.017.

References


