



Low nanomolar GABA effects at extrasynaptic $\alpha 4\beta 1/\beta 3\delta$ GABA_A receptor subtypes indicate a different binding mode for GABA at these receptors

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ABSTRACT

Ionotropic GABA_A receptors are a highly heterogeneous 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 $\alpha 4\beta 3\delta$ GABA_A receptors. $\alpha 4/\delta$ -Containing GABA_A receptors displayed high sensitivity to GABA, with mid-nanomolar concentrations activating $\alpha 4\beta 1\delta$ ($EC_{50} = 24$ nM) and $\alpha 4\beta 3\delta$ ($EC_{50} = 12$ nM) receptors. In the majority of oocytes expressing $\alpha 4\beta 3\delta$ subtypes, GABA produced a biphasic concentration-response curve, and activated the receptor with low and high concentrations ($EC_{50}(1) = 16$ nM; $EC_{50}(2) = 1.2$ μ M). At $\alpha 4\beta 2\delta$, GABA had low micromolar activity ($EC_{50} = 1$ μ M). An analysis of 10 N-terminal singly mutated $\alpha 4\beta 3\delta$ receptors shows that GABA interacts with amino acids different to those reported for $\alpha 1\beta 2\gamma 2$ GABA_A receptors. Residues Y205 and R207 of the $\beta 3$ -subunit significantly affected GABA potency, while the residue F71 of the $\alpha 4$ - and the residue Y97 of the $\beta 3$ -subunit did not significantly affect GABA potency. Mutating the residue R218 of the δ -subunit, equivalent to the GABA binding residue R207 of the $\beta 2$ -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 $\alpha 1$ – 6 , $\beta 1$ – 3 , $\gamma 1$ – 3 , δ , ϵ , θ , and π [1–3]. These subunits can mix and match to form possible heteromeric receptor subtypes. Of the six α -subunits, $\alpha 1$ – 3 are generally localized at synapses and mediate phasic inhibition, while $\alpha 4$ – 6 dominate sites outside the synapse and mediate tonic inhibition [4]. Receptors containing $\alpha 4$ – 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 $\alpha 4/\alpha 6$ -subunits. The $\alpha 6/\delta$ -containing receptors are located on cerebellar granule cells, while the $\alpha 4/\delta$ -containing receptors are present in the dentate gyrus, thalamus, and neostriatum [8]. Recently the δ -subunit was shown to colocalise with the $\alpha 1$ -subunit and as such, an $\alpha 1\beta\delta$ combination is thought to express at extrasynaptic sites on hippocampal interneurons [9].

Abbreviations: GABA, (γ -aminobutyric acid); (Zn^{2+}), Zinc.

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Given the distinct location of δ -subunit-containing GABA_A receptors, their high sensitivity to neurosteroids and lack of sensitivity to benzodiazepines, extrasynaptic 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_A receptors are important targets for drug development [19].

The potency of GABA at GABA_A 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_A 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 $\alpha 6\beta 3\delta$ GABA_A 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 $\alpha 4\beta 3\delta$ GABA_A 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 $\alpha\beta\gamma$ GABA_A 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 $\alpha 1\beta 2\gamma$ receptor subtype [26–29], are amino acids R207 and Y205 of the $\beta 2$ -subunit, and F64 of the $\alpha 1$ -subunit. In addition, residue Y97 of the $\beta 2$ -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 $\alpha 4\beta 1/\beta 3\delta$ GABA_A receptors, while at $\alpha 4\beta 2\delta$ GABA_A receptors, GABA is 100-fold weaker. Investigations into the molecular determinants for the nanomolar GABA effects at $\alpha 4\beta 3\delta$ GABA_A receptors revealed that residues, known to interact with GABA at $\alpha\beta\gamma$ GABA_A receptors, are not essential for $\alpha\beta\delta$ 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 $\alpha 1$ and $\beta 2$ in pcDM8 was provided by Dr Paul Whiting (Merck, Sharpe and Dohme Research Labs, Harlow, UK), $\beta 3$ in pGEMHE, $\alpha 4$, and δ in pcDNA1/Amp and $\alpha 6$ and $\beta 1$ in pcDM8 were a gift from Dr Bjarke 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 $\alpha 4$ - and $\beta 3$ -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_A 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., Broomall, PA, USA) with cRNA reconstituted in nuclease free water in a ratio of 1:1 for $\alpha 1:\beta 2/\beta 3$ except 5:1 for $\alpha 4/\alpha 6:\beta 3$, 1:1:5 for $\alpha 4:\beta 1/\beta 2:\delta$ and 5:1:5 for $\alpha 4:\beta 3:\delta$ receptors. For the expression of mutant receptors, a ratio of 5:1 for $\alpha 4:\beta 3$ and 5:1:5 for $\alpha 4:\beta 3:\delta$ 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 MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.4), supplemented with pyruvate (5 mM), gentamycin (50 $\mu\text{g}/\text{mL}$) and 2% horse serum at 18°C .

In the second independent study, the cRNA of the $\alpha 4$ -, $\beta 3$ - and δ -subunits was mixed in a 1:1:2 ratio to a final concentration of 0.5 $\mu\text{g}/\mu\text{l}$. The cRNA was injected into the stages V–VI oocytes obtained from Ecocyte (Ecocyte Bioscience, Castrop-Rauxel, Germany), and incubated for 5–8 days at 18°C in Modified Barth's Solution (90 mM NaCl, 1 mM KCl, 0.66 mM NaNO₃, 2.4 mM NaHCO₃, 0.82 mM MgCl₂, 0.74 mM CaCl₂, 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 M Ω). 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, Zn²⁺ 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 Zn²⁺ (0.1 and 1 μM) against GABA EC₅₀ 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 $I\% = (I/I_{\max}) \times 100$, where I is the peak amplitude of current response and I_{\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: $I = I_{\max}/(1 + [EC_{50}/A]^{n_H})$; alternatively, the data were fitted with a sum of two Hill equations

$$I = \frac{I_{\max}^1}{(1 + [EC_{50}^1/[A]]^{n_{H1}})} + \frac{I_{\max}^2}{(1 + [EC_{50}^2/[A]]^{n_{H2}})}$$

where I is the peak amplitude of the current elicited by a given concentration of agonist $[A]$, I_{\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 v5 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 Dunnett'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 δ -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 δ -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 μM Zn^{2+} was able to inhibit the current produced by GABA (10 μM) 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 μM) was able to partially inhibit the response of GABA and only when the GABA concentration was increased to 3 μM , a concentration higher than the reported EC_{50} at this receptor [5]. Thus Zn^{2+} (1 μM) inhibited GABA (0.1 μM) by only $10 \pm 3\%$ ($n = 8$; Fig. 1B) while the same concentration of Zn^{2+} (1 μM) inhibited GABA (3 μM) by $40 \pm 5\%$ ($n = 8$; Fig. 1C and D). These data ascertained that ternary δ -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 μM (Table 1), with the

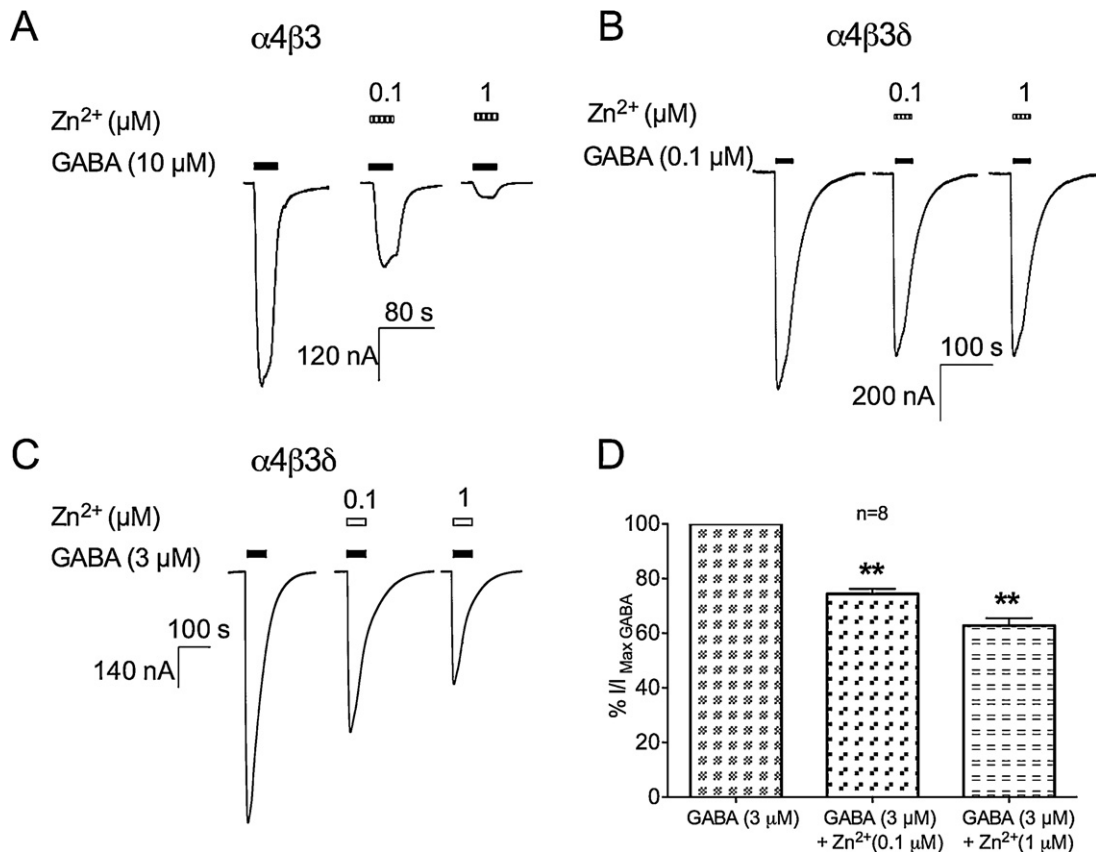


Fig. 1. Example of oocyte recordings (current (nA) vs time (s)) illustrating the GABA induced response and the effect of Zn^{2+} . Zn^{2+} (0.1 and 1 μM) inhibited (A) GABA (10 μM) at $\alpha 4\beta 3$ receptors by 55% and 95% respectively. (B) GABA (0.1 μM) at $\alpha 4\beta 3\delta$ receptors by 5% and 10% respectively. (C) GABA (3 μM) 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 μM) by Zn^{2+} (0.1 and 1 μM) at $\alpha 4\beta 3\delta$ receptors. Zn^{2+} (1 μM) significantly inhibited GABA (3 μM) by $38 \pm 3\%$ (ANOVA followed by Dunnett's post hoc test; $n = 8$; $**p < 0.01$ compared to GABA alone).

Table 1Effect of GABA on GABA_A receptors containing or not containing the δ -subunit.

Receptor subtype	GABA EC ₅₀ (μ M) (95% CI)	n_H	Holding current (mean \pm SEM) nA	<i>n</i>
$\alpha 1\beta 3$	34.5 (23.4–50.8)	0.8 \pm 0.3		3
$\alpha 4\beta 1$	0.72 (0.6 \pm 0.8)	1.0 \pm 0.2		6
$\alpha 4\beta 2$	2.3 (1.5–3.4)	0.9 \pm 0.3		4
$\alpha 4\beta 3$	0.52 (0.29–0.97)	0.6 \pm 0.2		6
$\alpha 6\beta 1$	7.7 (5.0–11.6)	1.2 \pm 0.2		4
$\alpha 6\beta 2$	2 (1.4–2.8)	1.3 \pm 0.3		4
$\alpha 6\beta 3$	1.7 (0.8–3.5)	0.5 \pm 0.2		4
$\alpha 1\beta 3\delta$	8.7 (6.4–11.7)	0.8 \pm 0.3		4
$\alpha 6\beta 1\delta^a$	0.35 (0.24–0.52)	0.9 \pm 0.3	–110 \pm 15	6
$\alpha 6\beta 2\delta^a$	(1) = 0.05 (0.02–0.15) (2) = 8.7 (3.7–20)	(1) = 0.9 \pm 0.3 (2) = 1.7 \pm 1.0	–180 \pm 30	4
$\alpha 6\beta 3\delta^a$	0.44 (0.33–0.53)	0.9 \pm 0.4	–80 \pm 20	10
$\alpha 4\beta 1\delta^a$	0.024 (0.019–0.030)	1.1 \pm 0.1	–350 \pm 35	8
$\alpha 4\beta 2\delta^a$	1.00 (0.89–1.31)	1.3 \pm 0.2	–60 \pm 20	4
$\alpha 4\beta 3\delta$ biphasic 5:1:5 injection ratio ^{a,b}	(1) ^b = 0.012 (0.006–0.025) (2) ^b = 1.3 (0.7–2.5)	(1) ^b = 1.2 \pm 0.5 (2) ^b = 1.1 \pm 0.3	–675 \pm 65	24
$\alpha 4\beta 3\delta$ biphasic 1:1:2 injection ratio ^{a,c}	(1) ^c = 0.008 (0.004–0.016) (2) ^c = 1.6 (0.5–4.9)	(1) ^c = 0.9 \pm 0.3 (2) ^c = 1.5 \pm 1.1		8
$\alpha 4\beta 3\delta$ monophasic ^{a,b}	0.016 (0.014–0.018)	0.8 \pm 0.1		5

^a Constitutively active GABA_A receptor subtypes.^b Data obtained at the Faculty of Pharmacy, the University of Sydney Australia.^c Data obtained from Neurosearch A/S, Ballerup, Denmark.

$\alpha 4\beta 3$ subtype being the most sensitive to GABA (Fig. 2A; Table 1; EC₅₀ = 520 nM; n_H = 0.6 \pm 0.2).

In contrast, GABA_A receptors formed from $\alpha 4/\alpha 6\beta 1\delta$, $\alpha 4\beta 2\delta$ and $\alpha 1/\alpha 6\beta 3\delta$ 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 $\alpha 4\beta 1\delta$ (Table 1; Fig. 2B; EC₅₀ = 24 nM; 95% CI 19–30 nM) and $\alpha 4\beta 3\delta$ (EC₅₀ = 16 nM; 95% CI 14–18 nM (Table 1; Fig. 3; see also Section 3.2 and Supplementary information Fig. S2) and the lowest potency displayed for the $\alpha 1\beta 3\delta$ GABA_A subtype (EC₅₀ = 8.7 μ M; Table 1; p < 0.05; ANOVA followed by Tukey's post hoc test). The EC₅₀ of GABA at $\alpha 4\beta 2\delta$, $\alpha 6\beta 1\delta$ and $\alpha 6\beta 3\delta$ did not significantly differ (p > 0.05; ANOVA followed by Tukey's post hoc test) and varied between 0.35 and 1.0 μ M (Fig. 2B; Table 1). As reported by others [37], GABA activated $\alpha 6\beta 2\delta$ GABA_A subtypes in a concentration dependent manner producing a biphasic concentration-response curve (Supplementary information Fig. S3).

3.2. GABA exerts a biphasic response at $\alpha 4\beta 3\delta$ GABA_A receptors

In the majority of $\alpha 4\beta 3\delta$ -expressing oocytes (24 out of 29 oocytes), the concentration-response curve for GABA displayed a shallow Hill slope ($n_H \leq 0.6$) with an EC₅₀ of 0.4 μ M, similar to the EC₅₀ of GABA at $\alpha 4\beta 3$ receptors (Fig. 3A). The low Hill slope (n_H) for $\alpha 4\beta 3\delta$ -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 EC₅₀ value for GABA at the $\alpha 4\beta 3\delta$ subtype was 12 nM (95% CI: 6–25 nM), while the low micromolar EC₅₀ value for GABA was 1.3 μ M (95% CI: 0.7–2.5 μ M). The latter value was not significantly different to the EC₅₀ of GABA at binary $\alpha 4\beta 3$ receptors (Student's t -test; p = 0.07). Given that Zn²⁺ can inhibit a high concentration of GABA at $\alpha 4\beta 3\delta$ receptors, it could be inferred that the biphasic response curve was due to a mixed population of receptors composed of $\alpha 4\beta 3\delta$ and $\alpha 4\beta 3$ GABA_A receptors. Furthermore, injection of $\alpha 4$ and δ mRNA alone and $\alpha 4$ and δ together did not form functional homomeric or heteromeric GABA gated channels (data not shown).

In 5 out of 29 oocytes expressing $\alpha 4\beta 3\delta$ GABA_A receptors, the Hill slope was 0.8 \pm 0.1 (Table 1; Fig. 3C) and data were preferentially fitted to a one-site model. The EC₅₀ value for GABA was 16 nM, approximately 32 times lower than the EC₅₀ value obtained for GABA

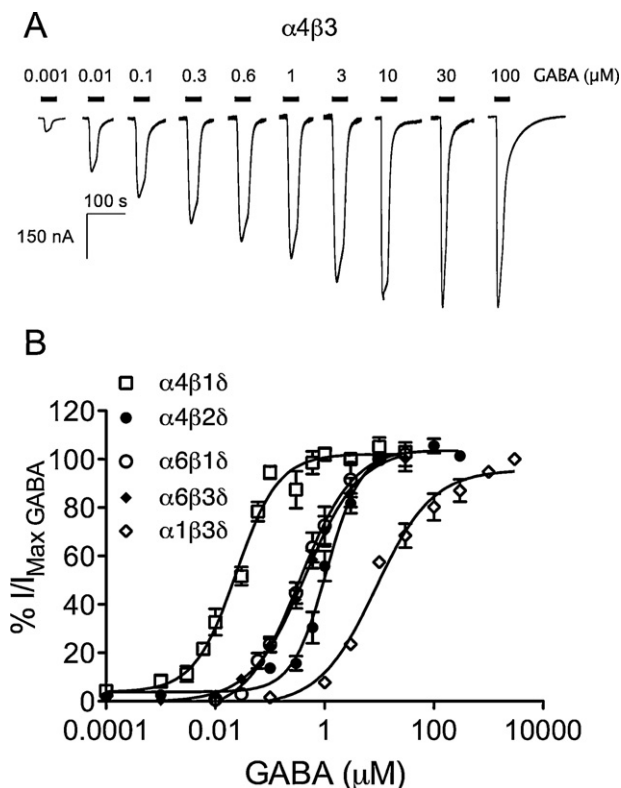


Fig. 2. (A) Example of an oocyte trace showing concentration dependent GABA responses $\alpha 4\beta 3$. (B) Concentration-response curves for GABA at $\alpha 4\beta 1\delta$ (\square , n = 8), $\alpha 4\beta 2\delta$ (\bullet , n = 4), $\alpha 6\beta 1\delta$ (\circ , n = 6), $\alpha 6\beta 3\delta$ (\blacklozenge , n = 10), and $\alpha 1\beta 3\delta$ (\diamond , n = 4) GABA_A receptor subtypes.

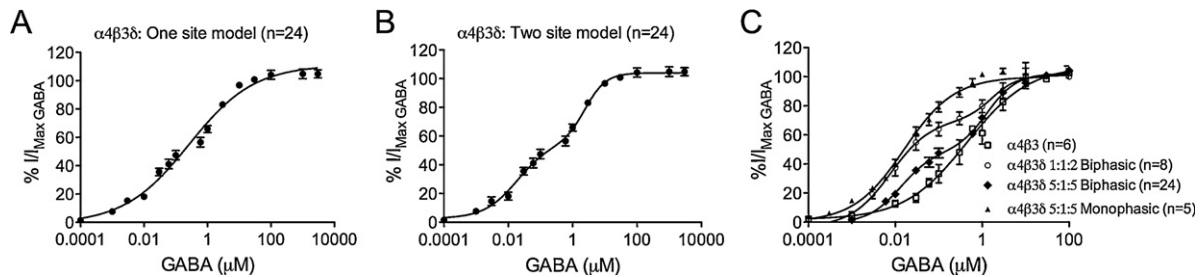


Fig. 3. (A) Concentration–response curve for GABA at $\alpha 4\beta 3\delta$ receptors ($n = 24$) fitted with a one-site model. (B) Concentration–response curve for GABA at $\alpha 4\beta 3\delta$ receptors fitted with a two-site model ($n = 24$). (F (DFn, DFd) 9.028 (3, 150)). Data are expressed as mean \pm SEM ($n = 24$). (C) Concentration–response curves for GABA at $\alpha 4\beta 3$ (\square , $n = 6$), $\alpha 4\beta 3\delta$ injected with a 5:1:5 cRNA ratio and fitted to a one-site model (\blacktriangle , $n = 5$), $\alpha 4\beta 3\delta$ injected with a 5:1:5 cRNA ratio and fitted to a two-site model (\blacklozenge , $n = 24$) and $\alpha 4\beta 3\delta$ cRNA injected with a 1:1:2 ratio and fitted to a two-site model (\circ , $n = 8$).

at $\alpha 4\beta 3$ receptors and was not significantly different from the low-to-mid-nanomolar EC_{50} value obtained from the biphasic curve. An example of an oocyte expressing a single population of receptors composed of $\alpha 4\beta 3\delta$ GABA_A receptor 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 $\alpha 4$, $\beta 3$, δ was 1:1:2 and different to our 5:1:5 injection ratio. Varying the injection ratio of cRNA enabled the fraction of expressed receptors. This approach is extensively used for studying the various stoichiometric forms of nicotinic acetylcholine $\alpha 4\beta 2$ receptor subtypes [38]. When a 1:1:2 injection ratio was used, a $70 \pm 10\%$ “high” versus $30 \pm 10\%$ “low” potency receptor fraction was observed (Fig. 3C). In contrast, when a 5:1:5 injection ratio was used, there was a $45 \pm 10\%$ “high” versus a $55 \pm 10\%$ “low” potency receptor population. The EC_{50} values obtained for the 1:1:2 injection ratio ($n = 6$) were 8 nM (95% CI: 4–16 nM) and 1.6 μ M (95% CI: 0.5–4.9 μ M), respectively, and were not significantly different to the EC_{50} 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 $\alpha 4\beta 3$ GABA_A receptors

The dramatic increase in GABA potency at $\alpha 4\beta 3\delta$ 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 $\alpha 4$ - and $\beta 3$ - for $\alpha 4\beta 3$ GABA activity. Specifically, mutant subunits $\alpha 4F71L$, $\beta 3Y205A$, $\beta 3R207A$ and $\beta 3Y97A$ were generated which correspond to $\alpha 1F64$ [26], $\beta 2Y205$, $\beta 2R207$ [27,29] and $\beta 2Y97$ [28,30].

The specific binary combinations included, $\alpha 4\beta 3Y97A$, $\alpha 4\beta 3Y205A$, $\alpha 4\beta 3R207A$ and $\alpha 4F71L\beta 3$ GABA_A receptors. As shown in Fig. 4, GABA activated currents in a concentration-dependent manner at all binary combinations. Examples of a GABA trace for $\alpha 4F71L\beta 3$ and $\alpha 4\beta 3R207A$ 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 $\alpha 4\beta 3$ receptors (Fig. 4C; Table 2). The EC_{50} values for GABA were 11.5-, 4410-, 1040- and 24 times higher at $\alpha 4\beta 3Y97A$, $\alpha 4\beta 3Y205A$, $\alpha 4\beta 3R207A$ and $\alpha 4F71L\beta 3$ than at wildtype $\alpha 4\beta 3$ receptors, respectively.

3.4. Molecular determinants for GABA sensitivity at ternary $\alpha 4\beta 3\delta$ GABA_A 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 Zn^{2+} inhibition as a proof for δ -subunit incorporation (Supplementary information Fig. S4).

At two of the mutated receptors ($\alpha 4F71L\beta 3\delta$ (Fig. 5A) and $\alpha 4\beta 3Y97A\delta$), GABA was only slightly affected, with 5.5- and 11.5-fold weaker potency relative to their wildtype counterparts. In contrast, mutating the $\beta 3$ 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

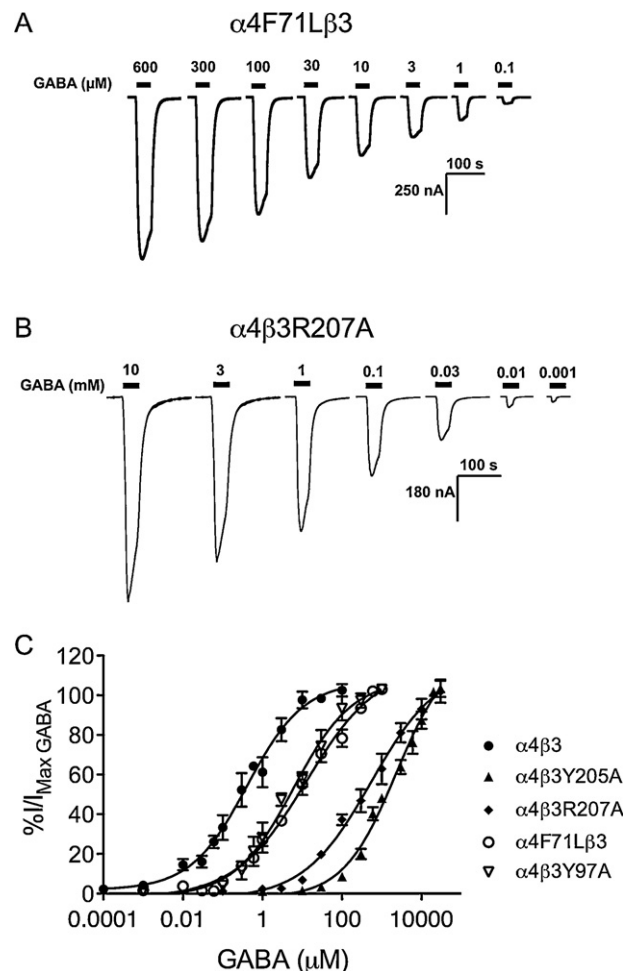


Fig. 4. Representative current traces showing the effect of GABA at binary (A) $\alpha 4F71L\beta 3$; (B) $\alpha 4\beta 3R207A$ mutant GABA_A receptors. Horizontal bars represent the duration of drug application. (C) Concentration–response curves for GABA at wild-type binary combinations for $\alpha 4\beta 3$ (\bullet), $\alpha 4\beta 3Y205A$ (\blacktriangle), $\alpha 4\beta 3R207A$ (\blacklozenge), $\alpha 4F71L\beta 3$ (\circ) and $\alpha 4\beta 3Y97A$ (∇) mutant receptors. Data represent mean \pm SEM ($n = 4$ –8).

Table 2Effects of N-terminal loop mutations on GABA potency at $\alpha 4\beta 3$ and $\alpha 4\beta 3\delta$ GABA_A receptors.

Receptor subtypes	EC ₅₀ (μ M) (95% CI)	n_H	EC ₅₀ (mutant)/EC ₅₀ (wildtype)	Holding current (mean \pm SEM) nA ^b	n
$\alpha 4\beta 3$	0.52 (0.29–0.97)	0.6 \pm 0.2	–	–	6
$\alpha 4F71L\beta 3$	12.7 (7.9–20.5) [*]	0.5 \pm 0.1	24	–	4
$\alpha 4\beta 3Y97A$	6.0 (3.2–11.3) [*]	0.6 \pm 0.1	11.5	–	4
$\alpha 4\beta 3Y205A$	2293 (1298–4051) ^{***}	0.7 \pm 0.1	4410	–	4
$\alpha 4\beta 3R207A$	540 (273–1066) ^{***}	0.6 \pm 0.1	1040	–	4
$\alpha 4\beta 3\delta^a$	0.016 (0.014–0.018)	0.8 \pm 0.1	–	–675 \pm 65	5
$\alpha 4F71L\beta 3\delta$	0.08 (0.07–0.10) ^{ns}	0.9 \pm 0.3	5	–220 \pm 60	4
$\alpha 4\beta 3Y97A\delta$	0.17 (0.08–0.32) ^{ns}	0.6 \pm 0.1	10	–260 \pm 70	4
$\alpha 4\beta 3Y205A\delta$	639 (454–900) ^{***}	0.8 \pm 0.2	39,940	–410 \pm 55	5
$\alpha 4\beta 3R207A\delta$	197 (152–256) ^{**}	0.8 \pm 0.3	12,310	–350 \pm 49	4
$\alpha 4\beta 3\delta R218A$	10.7 (7.1–16.2) [*]	0.6 \pm 0.1	670	–235 \pm 49	8

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

(Fig. 5C; Table 2). These data indicate that the Y205 and R207 residues are important for binding and/or gating.

From published data on concatenated $\alpha 6\beta 3\delta$ receptors, it is tempting to speculate that an alternative interface could exist for the low nanomolar potency of GABA at $\alpha 4\beta 3\delta$ receptors [24]. Thus

we examined the homologous residue to $\beta 3R207$ on the δ -subunit, $\delta R218$, and evaluated the potency of GABA when the residue was mutated to alanine. At $\alpha 4\beta 3\delta R218A$ 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 $\delta R218$ 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_A receptors expressed in oocytes. In addition, we used $\alpha 4\beta(\delta)$ 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 ($\alpha 1\beta 3\delta$, $\alpha 4\beta 1-3\delta$ and $\alpha 6\beta 1-3\delta$) are highly sensitive to GABA. However from our studies, GABA was 50- to 100-fold more potent on $\alpha 4\beta 1/\beta 3\delta$ GABA_A receptor subtypes than on $\alpha 4\beta 2\delta$ subtypes and differed in that the potency of GABA at $\alpha 4\beta 1/\beta 3\delta$ GABA_A receptors was 10–100-fold more potent than previously reported [5]. Low GABA concentrations ranging from 0.1 to 0.8 μ M [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 $\alpha 4\beta 3\delta$ GABA_A 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_A 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 $\alpha 4\beta 3\delta$ 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 $\alpha 4\beta 3\delta$ GABA_A receptors with “high” and “low” potencies, indicating that more than one receptor population is expressed. This biphasic concentration–response curve to GABA may be explained in two ways: either

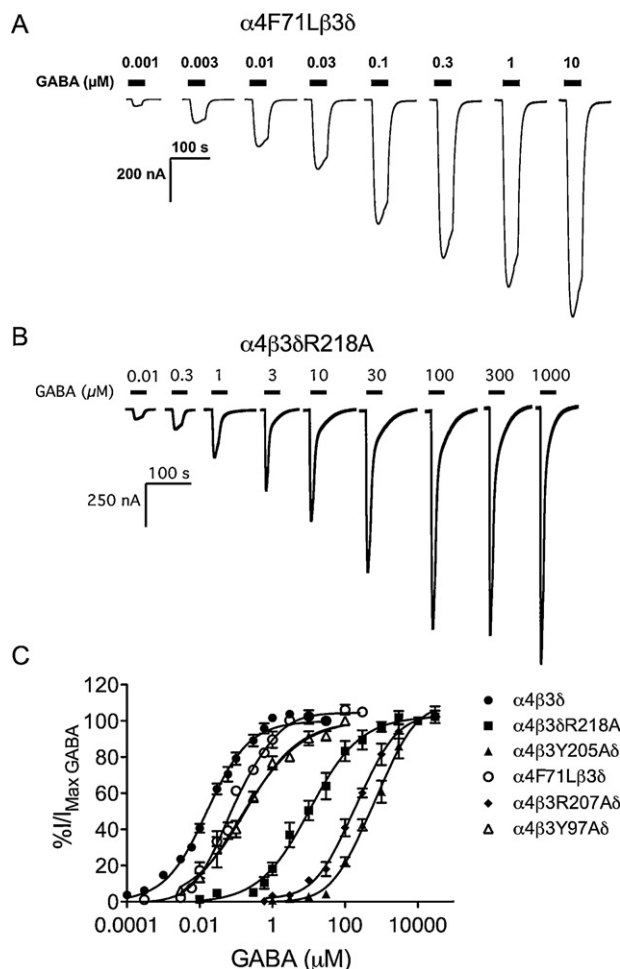


Fig. 5. Representative current traces showing the effect of GABA at ternary (A) $\alpha 4F71L\beta 3\delta$; and (B) $\alpha 4\beta 3\delta R218A$ mutant GABA_A receptors. Horizontal bars represent the duration of drug application. (C) Concentration–response curves for GABA at wildtype ternary combinations for $\alpha 4\beta 3\delta$ (●), $\alpha 4\beta 3\delta R218A$ (■); $\alpha 4\beta 3Y205A\delta$ (▲), $\alpha 4F71L\beta 3\delta$ (○), $\alpha 4\beta 3R207A\delta$ (◆), and $\alpha 4\beta 3Y97A\delta$ (△). Data represent mean \pm SEM ($n = 4–16$).

different stoichiometric forms or subunit arrangements for $\alpha 4\beta 3\delta$ GABA_A receptors are being formed or a mixture of both binary and ternary receptors is present.

Studies using concatenated receptors composed of $\alpha 1/\alpha 6$ -, $\beta 3$ - 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 $\alpha 4\beta 3\delta$ GABA_A 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 $\alpha 4$ - $\beta 2$ - $\alpha 4$ - $\beta 2$ - δ was reported indicating that this arrangement [and possibly others] is functional [47]. Should different stoichiometric forms or arrangements exist for $\alpha 4\beta 3\delta$ GABA_A 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 $\beta(+)$ - and $\alpha(-)$ -subunit. The counter-clockwise arrangement β - α - β - α - δ contains two $\beta(+)$ - and $\alpha(-)$ -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 $\alpha 6\beta 2\delta$ receptors with GABA and other agonists exhibiting distinct EC₅₀ values [37].

Whilst we cannot rule out the possibility that stoichiometric forms of $\alpha 4\beta 3\delta$ GABA_A receptors exist, there are several reasons to indicate that the “high” and “low” potency receptors are due to the expression of $\alpha 4\beta 3\delta$ and ‘contamination’ of $\alpha 4\beta 3$ GABA_A receptors: Zn²⁺ is a potent inhibitor of binary GABA_A receptors composed of $\alpha\beta$ subunits and in our studies Zn²⁺ partially inhibited the response of GABA, indicating that a population of the receptors may not contain the δ -subunit. Supporting this claim, Zn²⁺ inhibited more of the GABA response from singularly injected mRNA of $\alpha 4$ -, $\beta 2$ -, δ -subunits in oocytes, indicating mixed populations of binary and possibly homomeric receptors composed of $\beta 2$ -subunits, than the concatenated form of $\alpha 4$ - $\beta 2$ - δ receptors [47]; the potency of GABA

at the “low” potency site was not significantly different to the potency of GABA at binary $\alpha 4\beta 3$ GABA_A receptors; and the fraction of the “high” potency site can vary with the amount of $\alpha 4$ - and/or δ -subunit mRNA injected in oocytes (from 5:1:5 to 1:1:2). It is interesting to note that robust binary $\alpha 4\beta 3$ receptors were only observed when a 5:1 mRNA injection ratio was used. In addition, Meera and colleagues showed that gaboxadol (THIP) also activates $\alpha 4\beta 3\delta$ GABA_A receptors with “high” and “low” potencies [22]. They concluded that only the “high” potency response exhibited by gaboxadol (THIP) was due to ternary $\alpha 4\beta 3\delta$ and that the “low” potency response was due to binary $\alpha 4\beta 3$ GABA_A receptors, i.e. the δ -subunit was not fully incorporated.

In an attempt to further understand how GABA activates $\alpha 4\beta 3$ and $\alpha 4\beta 3\delta$ GABA_A 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 $\beta 2$ - and the residue F64 in $\alpha 1$ -subunits are all known to form part of the GABA binding site, located at the interface of the principal $\beta(+)$ - and complementary $\alpha(-)$ -subunits of synaptic $\alpha\beta\gamma$ receptor subtypes [26,27,29]. We found that the potency of GABA at $\alpha 4\beta 3$ and $\alpha 4\beta 3\delta$ receptors was strongly affected by mutating the corresponding amino acids $\beta 3$ R207 and $\beta 3$ Y205. Surprisingly, the potency of GABA was less dramatically affected than expected when the $\alpha 4$ F71 residue was mutated to alanine. There was a 24-fold reduction in the potency of GABA at $\alpha 4\beta 3$ but only a 5-fold reduction at $\alpha 4\beta 3\delta$ receptors. This indicates that GABA may have a different binding mode at $\alpha 4\beta 3$ and $\alpha 4\beta 3\delta$ receptors than at $\alpha 1\beta 2$ and $\alpha 1\beta 2\gamma 2$ GABA_A receptors where the corresponding mutation in the $\alpha 1$ -subunit right-shifts the GABA curve by 72- [28] and 210-fold [26], respectively. Thus F71 of the $\alpha 4$ -subunit may not significantly contribute to GABA binding at these receptors.

A similar observation was made for the $\beta 3$ Y97A mutant which did not dramatically affect GABA potency at either $\alpha 4\beta 3$ or $\alpha 4\beta 3\delta$ GABA_A receptors. This residue is situated on the principal (+) side of the β -subunit and has previously been shown (in $\beta 2$) to interact with the amino group of GABA via a cation- π interaction [30]. In fact, mutating $\beta 3$ Y97 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 $\alpha 1\beta 2\gamma 2$ receptors [28,30].

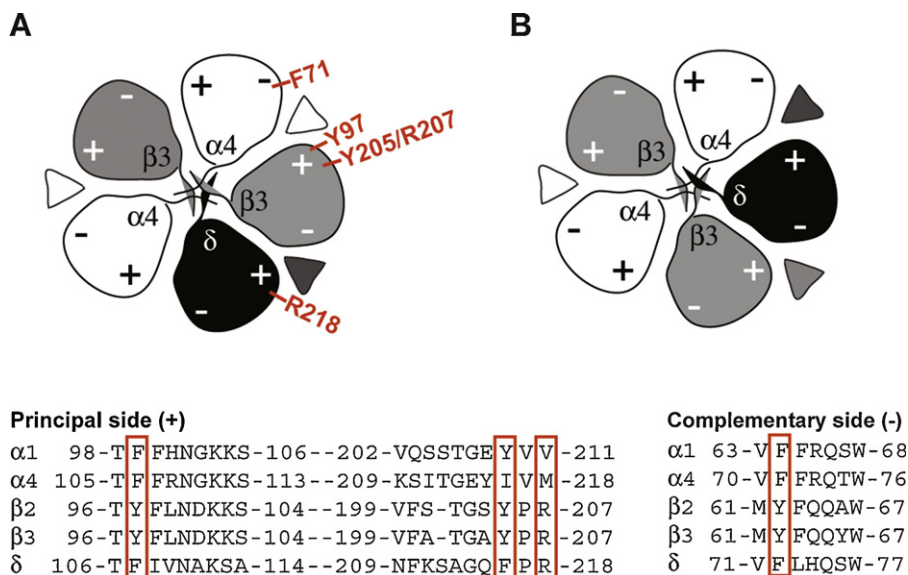


Fig. 6. Cartoon illustrating the possible arrangements for $\alpha 4\beta 3\delta$ as described by Barrera and colleagues [25]. Binding sites for GABA (triangles) are indicated as well as the studied mutations on the principal $\beta(+)$ or $\delta(+)$ sides or in the complementary $\alpha(-)$ interfaces. In the counter-clockwise arrangement β - α - β - α - δ (A), a GABA binding site may potentially exist at the $\delta(+)$ interface in addition to the two $\beta(+)$ -participating interfaces. In the counter-clockwise arrangement α - β - α - β - δ (B), three non-equivalent GABA sites are possible: two at the $\beta(+)$ -interfaces, and one at the $\delta(+)$ interface. The “high” and “low” potency effects exerted by GABA may arise from interaction at either of the two arrangements.

Interestingly the δ -subunit residue R218, corresponding to β R207 in sequence alignments (Fig. 6), was found to significantly affect GABA potency. The 670-fold reduction in potency of $\alpha 4\beta 3\delta$ R218A 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 $\alpha 4\beta 3\delta$ complex. Indeed studies using concatenated $\alpha 6\beta 3\delta$ 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 $\beta(+)$ -interface: as a $\delta(+)$ - $\beta 3(-)$ interface for the clockwise arrangement and a $\delta(+)$ - $\alpha 4(-)$ and/or a $\beta 3(+)$ - $\delta(-)$ interface for the anticlockwise arrangement.

The fact that mutations in the $\beta 3$ -subunit (Y205 and R207) have much greater effects on GABA potency than the δ R218 mutation suggests that GABA binding at the $\beta 3(+)$ - $\alpha 4(-)$ 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 δ R218 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 $\alpha 4\beta 1/\beta 3\delta$ GABA_A receptors and that differences in potency amongst research groups may, in part, be the result of varying fractions of binary $\alpha 4\beta 1/\beta 3$ and ternary $\alpha 4\beta 1/\beta 3\delta$ receptor expression. In addition, mutational analysis shows that the potency of GABA at $\alpha 4\beta 3\delta$ receptors is dependent on different amino acid residues at homologous sites involving $\beta(+)$ and $\delta(+)$ interface domains than those of $\alpha 1\beta 2$ and $\alpha 1\beta 2\gamma$ GABA_A 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 GABA_A 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>.

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