Potency of GABA at human recombinant GABA$_A$ receptors expressed in *Xenopus* oocytes: a mini review

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Abstract GABA$_A$ receptors are members of the ligand-gated ion channel superfamily that mediate inhibitory neurotransmission in the central nervous system. They are thought to be composed of 2 alpha (α), 2 beta (β) subunits and one other such as a gamma (γ) or delta (δ) subunit. The potency of GABA is influenced by the subunit composition. However, there are no reported systematic studies that evaluate GABA potency on a comprehensive number of subunit combinations expressed in *Xenopus* oocytes, despite the wide use of this heterologous expression system in structure–function studies and drug discovery. Thus, the aim of this study was to conduct a systematic characterization of the potency of GABA at 43 human recombinant GABA$_A$ receptor combinations expressed in *Xenopus* oocytes using the two-electrode voltage clamp technique. The results show that the α-subunits and to a lesser extent, the β-subunits influence GABA potency. Of the binary and ternary combinations with and without the γ2L subunit, the α6/γ2L-containing receptors were the most sensitive to GABA, while the β2- or β3-subunit conferred higher sensitivity to GABA than receptors containing the β1-subunit with the exception of the α2β1γ2L and α6β1γ2L subtypes. Of the δ-subunit containing GABA$_A$ receptors, α4/δ-containing GABA$_A$ receptors displayed highest GABA sensitivity, with mid-nanomolar concentrations activating α4β1δ and α4β3δ receptors. At α4β2δ, GABA had low micromolar activity.

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Introduction

γ-Aminobutyric acid (GABA), the principal inhibitory neurotransmitter in the vertebrate central nervous system (CNS) mediates fast synaptic transmission via ionotropic GABA$_A$ receptors. These receptors are pentameric assemblies of individual subunits arranged around a central, chloride-conducting pore. To date, 16 human GABA$_A$ receptor subunit genes have been characterized and grouped together according to their amino acid similarity and termed: α1–6, β1–3, γ1–3, δ, ϵ, θ, and π (Bonnert et al. 1999; Barnard et al. 1998; Sinkkonen et al. 2000). Of the six α-subunits, α1–3 are localized at synapses, while α4–6 subunits exist at sites outside the synapse (Belelli et al. 2009), and have higher sensitivity to GABA (Storustovu and Ebert 2006). The activation of GABA$_A$Rs by GABA is therefore not restricted to fast synaptic transmission alone, and is in part dependent on subunit composition.

The γ2-containing GABA$_A$ receptors can be found at both synaptic and extrasynaptic sites. These γ2-subunits combine with α1–3,5-subunits to form receptors that are sensitive to benzodiazepines (Rudolph et al. 2001; Atack et al. 2005). Each α-subunit confers different physiological

characteristics, and displays differential regional expression profiles in the brain. The role of each α-subunit in relation to benzodiazepine pharmacology has been elucidated using genetically modified mice in which a genetic mutation was introduced to individual α-subunits in order to render the receptor subtype insensitive to diazepam. These studies found that the sedative, anterograde, amnestic, and partly the anticonvulsant actions of diazepam were mediated by α1-containing GABA_A receptors, the anxiolytic-like effects were mediated by α2-containing GABA_A receptors, and the myorelaxant action was mediated in part by α3- and α5-containing GABA_A receptors. Moreover, the development of tolerance to the sedative action of benzodiazepines was linked to α5-containing GABA_A receptors, while the addictive properties have been linked to α1-containing GABA_A receptors (Rudolph et al. 2001).

The δ-subunit predominates on peri- and extrasynaptic locations (Nusser et al. 1998; Wei et al. 2003). Preferentially forming receptors with α4- or α6-subunits, the α6/δ-containing receptors are located to cerebellar granule cells, while the α4/δ-containing receptors are located to the dentate gyrus, thalamus, and neostriatum (Barnard et al. 1998). In addition to these combinations, binary GABA_A receptors composed of α- and β-subunits are thought to also exist in the CNS.

Determining which of these combinations has a distinct physiological role in the CNS has been hampered by the lack of subtype selective agents. Studies using a variety of techniques including in situ hybridization, immunoprecipitation and immunocytochemistry studies, and the development of transgenic mice, have enabled one to infer the likeliest native GABA_A receptor subtypes involved in distinct physiological roles (Wisden et al. 1992; Pirker et al. 2000; Korpi et al. 2002; Sperk et al. 1997; Hutcheon et al. 2004; Olsen and Sieghart 2008). However, given there is a high level of structural diversity of potential GABA_A receptor subtypes, the impact of this diversity on GABA potency is not well known (Olsen and Sieghart 2008; Sieghart 2006). Indeed, many subunit combinations can form functional receptors when different subunits are expressed in vitro, and these consist of binary combinations composed from α- and β-subunits, ternary combinations composed of α-, β-, and either γ- or δ-subunits, and in some cases quaternary combinations exist (Sieghart 2006). Further impacting on this complex set of combinations, the effect of stoichiometry remains to be evaluated systematically. As a result of this diversity, the sensitivity to GABA must differ given the different receptor locations and other biochemical conditions. In a recent study, Mortensen et al. (2012) evaluated the effect of GABA expressed in human embryonic kidney (HEK293) cells to try to address this issue. This study was quite comprehensive and serves as a valuable reference for GABA responses in this cellular

expression system. Given that choice of cell expression system contributes to the potency of GABA and other ligands, and that there is a large body of evidence that the host heterologous expression system influences the function of the resulting receptors at the cell membrane (Palma et al. 2003), the current study attempts to supplement with pharmacological data obtained using another highly used cell expression system, namely the *Xenopuslaevis* oocyte expression system. The oocyte expression system is a very robust and widely used expression system to evaluate the effects of agents acting on GABA_A receptors, but surprisingly little is known about the differing effects of GABA on these receptors. The current study and mini review provides data on the potency of GABA at 43 human GABA_A receptor subtypes composed of α1–6, β1–3 alone and in combination with γ2L- or δ-subunits expressed in *Xenopus* oocytes.

Materials and methods

GABA and buffer supplements were all purchased from Sigma (St. Louis, MO, USA).

GABA receptor subunit constructs

Human α1, α2, β2 and γ2L DNA in pcDM8 were provided by Dr. Paul Whiting (Merck, Sharpe and Dohme Research Labs, Harlow, UK). α3 and β3 in pGEMHE, α5 in pcDNA3, α4 and δ in pcDNA1/Amp, and α6 and β1 in pcDM8 were a gift from Prof Bjarke Ebert (H. Lundbeck A/S, Valby, Denmark). cDNA vectors were linearized with the appropriate restriction endonucleases (Table 1) and capped transcripts were produced from linearized plasmids using the 'mMessage mMachine' T-7 transcript kit from Ambion (Austin, TX, USA). The quality of cRNA was determined by 0.5 % agarose gel electrophoresis. mRNA concentrations were measured by NanoDrop® ND-1000 UV–Vis Spectrophotometer. cRNA was diluted with nuclease-free water and stored at −80 °C.

Expression of recombinant GABA receptors in *Xenopus* oocytes

Oocyte preparation

The methods for oocyte harvesting and preparation have been described previously (Karim et al. 2011). In brief, stage 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 1:1:10 for α:β:γ, 5:1:5 for α:β:δ and 1:1 for α:β receptors. Oocytes were incubated for 4–8 days in standard

Table 1 The pcDNA vector, restriction digestion enzyme and mRNA polymerase used for each receptor subunit

Receptor subunit	Plasmid	Restriction enzyme	RNA polymerase
α1	pcDM8	Not1	T7
α2	pcDM8	Not1	T7
α3	pGEMHE	Nhe1	T7
α4	pcDNA1/Amp	Hpa1	T7
α5	pcDNA3	Not1	T7
α6	pcDM8	Sap1	T7
β1	pcDM8	Hpa1	T7
β2	pcDM8	Not1	T7
β3	pGEMHE	Nhe1	T7
γ2L	pcDM8	Not1	T7
δ	pcDNA1/Amp	Hpa1	T7

sterile-filtered ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 1.8 mM CaCl$_2$, 5 mM HEPES, pH 7.4), supplemented with theophylline (0.5 mM), pyruvate (5 mmol/L), gentamycin (50 µg/mL) and 2 % horse serum at 18 °C.

Electrophysiology

Currents were recorded using the two-electrode voltage clamp (TEVC) technique as described elsewhere (Hall et al. 2005). Oocytes were individually placed in a 150 µl chamber connected to a reservoir bottle containing ND96 solution. Glass microelectrodes were made with a tip resistance of 0.2–1.2 MΩ 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).

Responses to GABA were normalized as $I\% = (I/I_{max}) \times 100$, where I is the peak amplitude of current response and I_{max} is the maximal current produced by GABA measured in each individual cell. Normalized responses were pooled and graphed as the mean ± SEM from at least three oocytes from at least two different batches. Responses were fitted to the four-parameter logistic equation: $I = I_{max}/(1 + (EC_{50}/[A])^{n_H})$; 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 coefficient (Prism v5 GraphPad software, San Diego, CA, USA). All statistical calculations are presented as means standard error of the

mean (SEM) or as mean [95 % confidence intervals (CI)]. Groups were compared using one-way ANOVA followed by Tukey's or Dunnett's post hoc test.

Results

Validation of expression of αβγ and αβδ receptors in *Xenopus* oocytes

With GABA-response curves for ternary receptor constructs, it is ideal that little to no current being measured is flowing through αβ binary receptors. Zn^{2+} has been reported to display distinct inhibitory effects at αβ- versus αβγ-containing receptors (Smart et al. 1991; Storustovu and Ebert 2006; Hosie et al. 2003); thus, we initially investigated the effects of zinc ions (Zn^{2+}) at representative GABA$_A$ receptor subtypes. In all combinations tested, we evaluated the effect of Zn^{2+}: at α1β2 receptors, 10 and 100 µM Zn^{2+} inhibited approximately 40 ± 8 % and 70 ± 14 % of the current elicited by GABA (100 µM), respectively, whereas at α1β2γ2L receptors, there was no inhibition by 10 or 100 µM Zn^{2+} (Fig. 1a, b, respectively) indicating that the γ2L subunit was incorporated. At α1β3 receptors, 0.1 and 1 µM Zn^{2+} inhibited approximately 55 ± 5 % and 80 ± 12 % of the current elicited by GABA (30 µM), respectively ($n = 3$; Fig. 1c), whereas at α1β3δ receptors, Zn^{2+} did not inhibit the current produced by GABA (10 µM; Fig. 1d, $n = 4$), implicating that the δ-subunit was incorporated (ANOVA followed by Dunnett's post hoc test; $n = 4$; $p > 0.05$). Similarly, at α4β3 receptors, Zn^{2+} (0.1 and 1 µM) inhibited GABA (10 µM) by 55 ± 10 % and 95 ± 5 % respectively ($n = 4$; Fig. 1e), whereas at α4β3δ receptors, Zn^{2+} (0.1 and 1 µM) inhibited GABA (3 µM) by 35 ± 9 % and 40 ± 6 %, respectively ($n = 6$; Fig. 1f), indicating a mixed population of receptors (Karim et al. 2012). Thus, Zn^{2+} was used throughout the study to demonstrate incorporation of the γ- and δ-subunits (data not shown).

Current ranges elicited by a maximum GABA concentration at the various GABA$_A$ receptor subtypes

The maximal current amplitude for GABA when tested at different αβ combinations ranged from 150 to 750 nA for α1β1–3, 100–350 nA for α2β1–3, 100–470 nA for α3β1–3, 80–500 nA for α4β1–3, 100–390 nA for α5β1–3, and 500–3000 nA for α6β1–3. Similarly, the maximal current amplitudes for different αβγ2L ranged from 300 to 4000 nA for α1β1–3γ2L, 290–1200 nA for α2β1–3γ2L, 200–3500 nA for α3β1–3γ2L, 350–4000 nA for α4β1–3γ2L, 300–740 nA for α5β1–3γ2L, and 3500–7000 nA for α6β1–3γ2L. The maximal current amplitude ranged
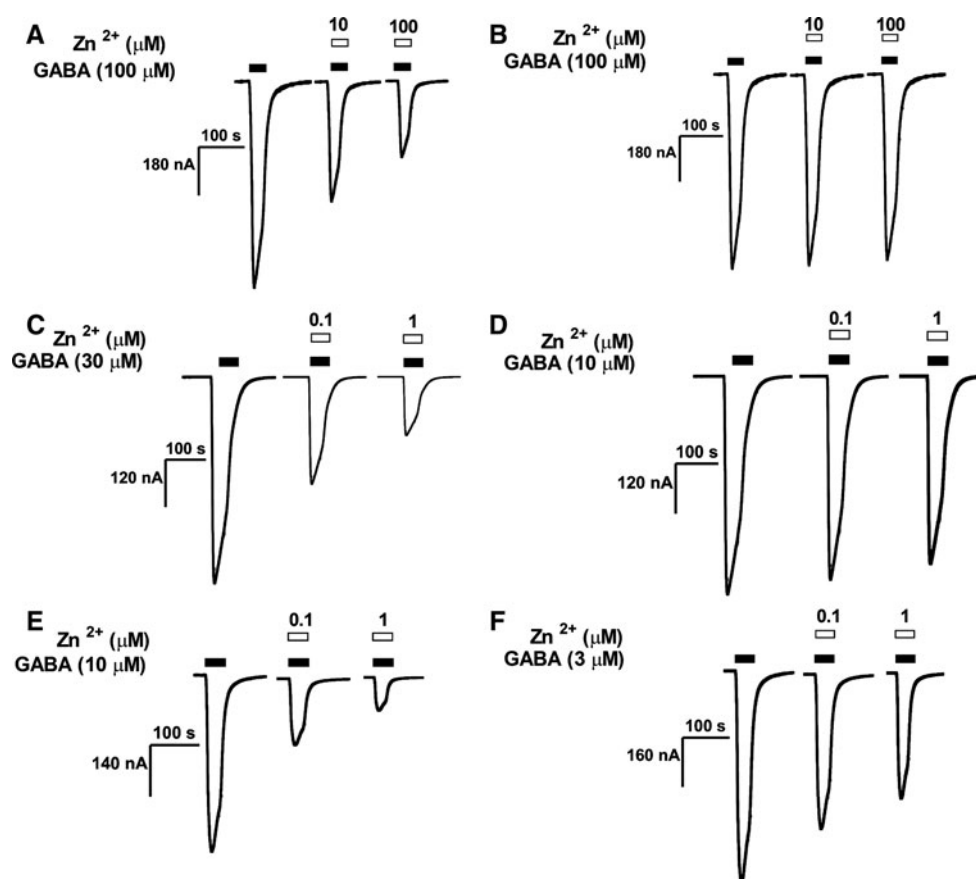
Fig. 1 Example of a recording from oocytes illustrating the blockade that Zn^{2+} exerts on the GABA response on $\alpha1\beta2$, $\alpha1\beta3$ and $\alpha4\beta3$ receptors expressed in *Xenopus* oocytes. **a**, **b** Current traces (nA vs. s) illustrating the blockade that Zn^{2+} exerts on the GABA response on $\alpha1\beta2$ and $\alpha1\beta2\gamma2L$ receptors. Zn^{2+} (10 and 100 μM) inhibited GABA (100 μM) by 40 $\pm$ 8 % and 70 $\pm$ 14 %, respectively, at $\alpha1\beta2$ receptors but did not inhibit GABA responses at $\alpha1\beta2\gamma2L$ receptors. **c**, **d** Zn^{2+} (0.1 and 1 μM) inhibited GABA (30 μM) by 55 $\pm$ 5 % and

80 $\pm$ 12 %, respectively, at $\alpha1\beta3$ receptors but did not inhibit GABA responses (10 μM) at $\alpha1\beta3\delta$ receptors. **e**, **f** Example of a trace showing the effect of Zn^{2+} inhibiting GABA at $\alpha4\beta3$ and $\alpha4\beta3\delta$ receptors. Zn^{2+} (0.1 and 1 μM) inhibited GABA (10 μM) by 55 $\pm$ 10 % and 95 $\pm$ 5 %, respectively, at $\alpha4\beta3$ and (0.1 and 1 μM) inhibited GABA (3 μM) by 35 $\pm$ 9 % and 40 $\pm$ 6 %, respectively, at $\alpha4\beta3\delta$ receptors

250–750, 500–1000, 550–4000, 500–4500, 3500–6500, 3500–6600, and 3500–7000 nA for $\alpha1\beta3\delta$, $\alpha4\beta1\delta$, $\alpha4\beta2\delta$, $\alpha4\beta3\delta$, $\alpha6\beta1\delta$, $\alpha6\beta2\delta$, and $\alpha6\beta3\delta$, respectively. The maximum chloride amplitude for each individual oocyte is dependent on the number of receptors expressed on the oocyte membrane, as well as the potential difference across the membrane, the chloride concentrations inside and outside the cell, and the intrinsic properties of the receptors including the single channel conductance and maximum open channel probability of the receptor. As such, it is not a reliable comparison of the efficiency of receptor expression at the cell surface for each subtype, but gives a guide to the relative ease of acquiring data for each subtype using the oocyte expression system.

Effect of GABA at $\alpha1$–$6\beta1$ and $\alpha1$–$6\beta1\gamma2L$ combinations

For studies of GABA$_A$ binary receptor combinations, all α-subunits were expressed sequentially with $\beta1$–3-subunits

and for ternary combinations sequentially with $\beta1$–3- and $\gamma2L$-subunits. The concentration–response curves for GABA at the $\alpha1$–$6\beta1$–3 and at $\alpha1$–$6\beta1$–$3\gamma2L$ receptors are shown in Fig. 2. GABA potency for each receptor combination was compared by determining the individual EC$_{50}$ value. The EC$_{50}$ and n_H values are summarized in Table 2. As previously reported, GABA was more potent on the binary combinations ($\alpha1$–$6\beta1$–3) than on the ternary combinations ($\alpha1$–$6\beta1$–$3\gamma2L$) (Ducic et al. 1995).

At the binary combinations containing the $\beta1$-subunit, GABA was most potent on $\alpha4\beta1$ and $\alpha6\beta1$ compared to other combinations (Fig. 2a, b). The EC$_{50}$ values varied between 0.7 and 268 μM. The apparent order of potency for the binary receptor subtypes is $\alpha4\beta1 > \alpha6\beta1 > \alpha5\beta1 > \alpha2\beta1 > \alpha1\beta1 > \alpha3\beta1$. The Hill coefficient ranged 0.6–1.5 for binary combinations ($\alpha1$–$6\beta1$–3) (Table 2). At the ternary GABA$_A$ receptor combinations, the EC$_{50}$ values varied between 18 and 260 μM, with GABA being the most potent at the $\alpha6\beta1\gamma2L$ receptor subtype (Fig. 2c, d).

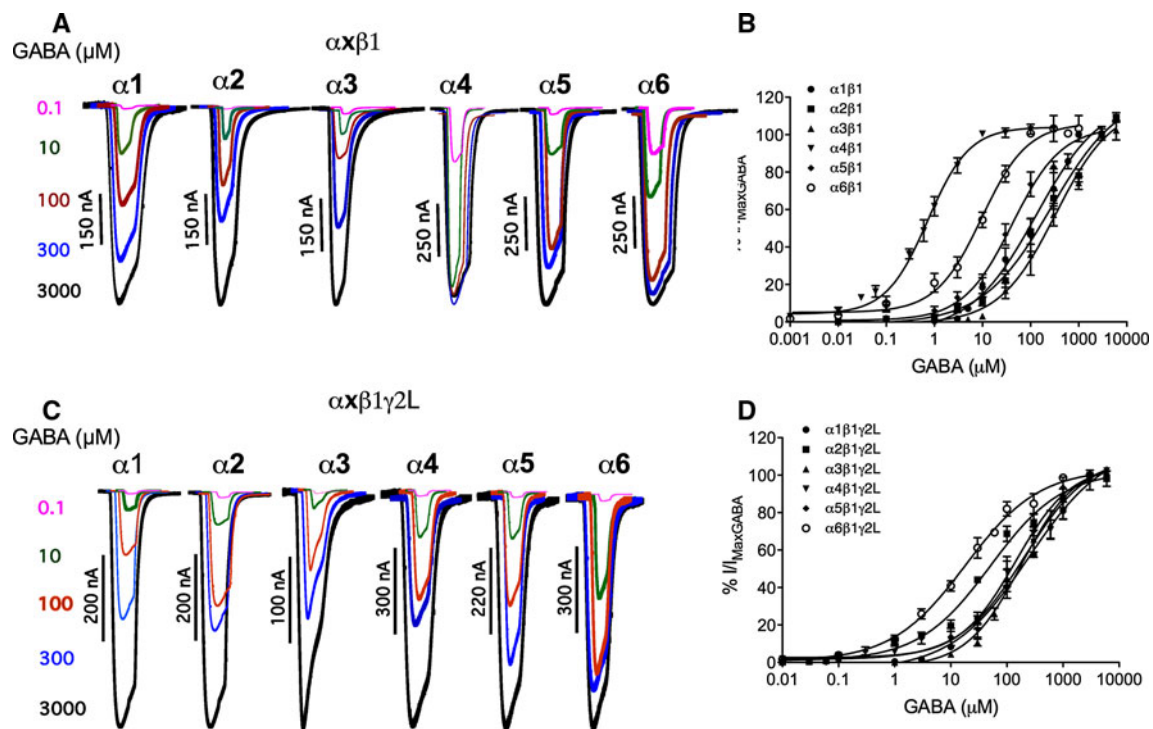**Fig. 2** Assessing the impact of α-subunits and the β1-subunit alone, and with the γ2L-subunit on GABA potency. **a** Superimposed membrane currents activated by GABA concentrations ranging from 0.1 μM to 3 mM for α1–6β1 GABA$_A$ receptors expressed in *Xenopus* oocytes. **b** Dose–response curves for GABA at α1β1 (*filled circle*) α2β1 (*filled square*) α3β1 (*filled upward triangle*) α4β1 (*filled downward triangle*) α5β1 (*filled diamond*) and α6β1 (*open circle*) receptor subtypes expressed

in *Xenopus* oocytes. **c** Superimposed membrane currents activated by GABA concentrations ranging from 0.1 μM to 3 mM for α1–6β1γ2L GABA$_A$ receptors expressed in *Xenopus* oocytes. **d** Dose–response curves for GABA at α1β1γ2L (*filled circle*), α2β1γ2L (*filled square*), α3β1γ2L (*filled upward triangle*), α4β1γ2L (*filled downward triangle*), α5β1γ2L (*filled diamond*) and α6β1γ2L (*open circle*) receptor subtypes expressed in *Xenopus* oocytes. Data are expressed as mean ± SEM ($n = 3$–10)

The order of potency for GABA at different ternary receptor subtypes is α6β1γ2L > α5β1γ2L > α2β1γ2L > α4β1γ2L > α3β1γ2L > α1β1γ2L. The Hill coefficient ranged 0.6–0.9 for ternary combinations (α1–6β1–3γ2L) (Table 2).

Effect of GABA at α1–6β2 and α1–6β2γ2L combinations

At the binary combinations containing the β2-subunit, GABA was found to be more potent on α4β2 and α6β2 combinations. The EC$_{50}$ values for α1–6β2 receptors varied between 2 and 56 μM (Table 2). The apparent order of potency is α6β2 > α4β2 > α1β2 ≈ α5β2 > α2β2 ≈ α3β2 (Fig. 3a, b; Table 2). The Hill coefficient ranged 0.6–1.3 for α1–6β2 binary subtypes. The EC$_{50}$ values for GABA at ternary receptor combinations containing the β2-subunit ranged from 25 to 133 μM. The apparent order of potency at these GABA$_A$ receptor subtypes is α6β2γ2L > α5β2γ2L > α4β2γ2L > α1β2γ2L > α2β2γ2L > α3β2γ2L (Fig. 3c, d; Table 2). The Hill coefficient ranged from 0.8 to 1.1 for α1–6β2 binary subtypes (Table 2).

Effect of GABA at α1–6β3 and α1–6β3γ2L combinations

At the binary combinations containing the β3-subunit, GABA was again found to be more sensitive compared to their ternary receptor subtype counterparts (Table 2). The EC$_{50}$ values varied between 0.4 and 90 μM at the binary receptor combinations. GABA was most sensitive to the α4β3 subtype with an EC$_{50}$ in the nanomolar range (0.41 μM), followed by α6β3 with an EC$_{50}$ of 1.6 μM (Fig. 4a, b; Table 1). The apparent order of potency at the binary subtypes was as follows α4β3 ≈ α6β3 > α5β3 > α1β3 > α2-β3 ≈ α3β3. The Hill coefficient for α1–6β3 subtypes ranged from 0.5 to 0.9 (Table 2).

GABA activated the ternary combinations with similar potencies ($p > 0.05$: ANOVA followed by Tukey's multiple comparison post hoc test), and the EC$_{50}$ values varied between 23 and 157 μM (Fig. 4c, d). The apparent order of potency for GABA is α6β3γ2L > α5β3γ2L > α3β3-γ2L ≈ α4β3γ2L > α1β3γ2L ≈ α2β3γ2L (Table 2). The Hill coefficient ranged 0.8–1.1 for α1–6β1–3γ2L ternary subtypes (Table 2).

Table 2 Agonist properties of GABA across α, β and γ GABA$_A$ receptor subunits

Receptor subtype	GABA EC$_{50}$ (μM) (95 % CI)	n_H	n
α1β1	141 (51.9–381)	0.7 ± 0.3	4
α2β1	45.4 (16.6–49.2)	1.5 ± 0.7	3
α3β1	268 (146–491.)	0.6 ± 0.3	3
α4β1	0.72 (0.60–0.80)	1.0 ± 0.2	5
α5β1	41.8 (29.8–58.5)	0.9 ± 0.2	5
α6β1	7.7 (5.0–11.6)	1.2 ± 0.2	4
α1β1γ2L	259 (179–375)	0.7 ± 0.3	6
α2β1γ2L	51.3 (32.6–80.7)	0.8 ± 0.5	4
α3β1γ2L	249 (64.2–193)	0.6 ± 0.5	5
α4β1γ2L	80 (51.5–127)	0.9 ± 0.2	6
α5β1γ2L	31.1 (23.4–41.2)	0.7 ± 0.3	6
α6β1γ2L	18 (13.2–25.1)	0.8 ± 0.4	5
α1β2	22 (14.4–32.5)	0.7 ± 0.3	6
α2β2	56 (26.9–119)	0.6 ± 0.3	3
α3β2	56.2 (34.8–90.7)	0.7 ± 0.5	3
α4β2	2.29 (1.54–3.40)	0.9 ± 0.3	4
α5β2	23.6 (14.6–38.0)	0.6 ± 0.3	5
α6β2	2 (1.4–2.8)	1.3 ± 0.3	4
α1β2γ2L	107 (74.2–156)	1.0 ± 0.2	8
α2β2γ2L	131 (93.8–182)	0.8 ± 0.3	10
α3β2γ2L	133 (88–201)	1.1 ± 0.2	6
α4β2γ2L	103 (61.9–173)	0.8 ± 0.3	6
α5β2γ2L	41.1 (34.2–49.2)	1.0 ± 0.2	5
α6β2γ2L	25 (18.8–33.2)	0.9 ± 0.3	6
α1β3	34.5 (23.4–50.8)	0.8 ± 0.3	3
α2β3	89.7 (44.3–182)	0.7 ± 0.4	3
α3β3	90.7 (44.3–183)	0.7 ± 0.5	4
α4β3	0.41 (0.25–0.69)	0.6 ± 0.1	8
α5β3	14.6 (10.4–20.4)	0.8 ± 0.3	5
α6β3	1.7 (0.8–3.5)	0.5 ± 0.2	4
α1β3γ2L	156 (96.0–252)	0.9 ± 0.2	5
α2β3γ2L	157 (94.9–259)	0.8 ± 0.4	5
α3β3γ2L	127 (81.7–206)	0.9 ± 0.2	4
α4β3γ2L	127 (68.2–236)	1.0 ± 0.2	6
α5β3γ2L	33.3 (22.4–49.3)	0.6 ± 0.5	5
α6β3γ2L	23.3 (15.5–34.9)	0.9 ± 0.3	8

In summary, the binary combinations containing α1–6β1–3 have higher GABA potencies compared to the ternary combinations containing α1–6β1–3γ2L.

Effect of GABA at recombinant GABA$_A$ receptors containing the δ-subunit

α4βδ and α6βδ receptors constitute the majority of extrasynaptic GABA$_A$ receptors (Boileau et al. 2002). Recently, we have investigated the effect of GABA at various δ-subunit containing GABA$_A$ receptors (Karim et al.

2012). Table 3 summarises the results from that study. Figure 5a shows the examples of GABA traces at α4β1δ, α4β2δ, α6β1δ, and α6β3δ GABA$_A$ receptors displaying mono-phasic concentration response curves. In contrast, α4β3δ (injected as a 5:1:5 mRNA ratio) and α6β2δ receptors displayed bi-phasic concentration response curves highlighting high and low affinity sites (Fig. 5b, c). GABA displayed the highest potency for α4β1δ (EC$_{50}$ = 0.024 μM), while potency 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 μM (Fig. 5d; Table 3).

Discussion

The pharmacological properties observed among the various native GABA$_A$ receptor subtypes are most likely related to specific mechanisms regulating receptor subunit expression (Wisden et al. 1992). Without selective agonists or antagonists, it is almost impossible to determine the properties of each native GABA$_A$ receptor subtype and identify which of the potential combinations exist, in vivo, let alone their changes in spatial and temporal expression.

Analysis of the effect of GABA at recombinant GABA$_A$ receptors has previously been described, but in many cases, for only a limited number of receptor subtypes or for different cell expression systems (Ducic et al. 1995; Kleingoor et al. 1993; Knoflach et al. 1996; Mortensen et al. 2012). As the cell expression system influences the effect of GABA, the current study focused on the *Xenopus* oocyte expression system because this system is extensively used for a number of structure–function studies and in drug discovery. A total of 43 human GABA$_A$ receptors were expressed in the oocyte system and the effect of GABA was evaluated using TEVC methods.

In receptors consisting of binary α- and β-subunits, or ternary α-, β- and γ2-subunits, the potency of GABA varied, influenced mainly by the type of α-subunit. In general, binary receptor subtypes were more sensitive to GABA than their γ2-containing counterparts. Binary GABA$_A$ receptors composed of β2- and β3-subunits did not affect GABA potency. GABA was most potent at receptors containing α4- and α6-subunits followed by α5- and α1-subunits. The least sensitive receptors to GABA were those composed of α2- and α3-containing receptors. In contrast, binary receptors composed of β1-subunit, GABA was most potent at α4- and α6-containing receptors but least potent at α3- and α1-containing receptors.

The marked differences in GABA potency may, in part, be related to the receptor stoichiometry. Indeed, the potency of acetylcholine can differ depending on the number of α- versus β-subunits that make up the
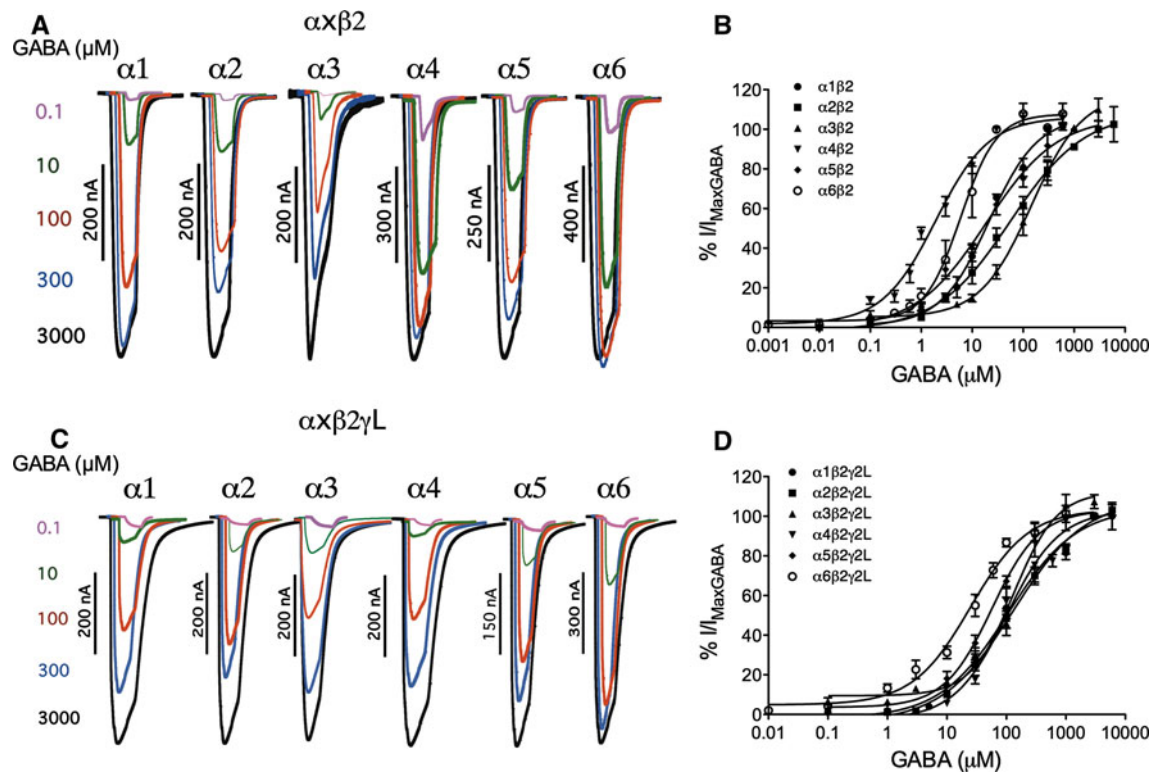
Fig. 3 Assessing the impact of α-subunits and β2-subunit alone, and with γ2L-subunit on GABA potency. **a** Superimposed membrane currents activated by GABA concentrations ranging from 0.1 μM to 3 mM for α1–6β2 GABA$_A$ receptors expressed in *Xenopus* oocytes. **b** Dose–response curves for GABA at α1β2 (*filled circle*) α2β2 (*filled square*) α3β2 (*filled upward triangle*) α4β2 (*filled downward triangle*) α5β2 (*filled diamond*) and α6β1 (*open circle*) receptor subtypes expressed in *Xenopus*

oocytes. **c** Superimposed membrane currents activated by GABA concentrations ranging from 0.1 μM to 3 mM for α1–6β2γ2L GABA$_A$ receptors expressed in *Xenopus* oocytes. **d** Dose–response curves for GABA at α1β2γ2L (*filled circle*), α2β2γ2L (*filled square*), α3β2γ2L (*filled upward triangle*), α4β2γ2L (*filled downward triangle*), α5β2γ2L (*filled diamond*) and α6β2γ2L (*open circle*) receptor subtypes expressed in *Xenopus* oocytes. Data are expressed as mean $\pm$ SEM ($n = 3$–10)

heteromeric nicotinic acetylcholine (nACh) receptor (Harpsoe et al. 2011; Wu et al. 2006). Furthermore, Wagoner and Czajkowski (2010) show that differences exist in the expression ratios of α- versus β-subunits for α1β2 and α4β2 GABA$_A$ receptors. In their study, a 3:2 stoichiometry was identified for α1β2, while for α4β2 GABA$_A$ receptors, the 2:3 stoichiometry was formed. How such differences in receptor stoichiometry could influence GABA potency is not known.

Our results and those of others (Mortensen et al. 2012; Ducic et al. 1995; Knoflach et al. 1996) show that among the ternary γ2L-containing receptor subtypes, the α6-containing receptors are the most sensitive to GABA, followed by the α5-containing receptors. In contrast to data obtained using HEK293 cells (Mortensen et al. 2012), we find that the least sensitive receptors are those composed of α1–4-containing receptors. Using oocytes, GABA potency did not significantly differ between α1–4-containing receptors, while using HEK293 cells, the α2- and α3-containing receptors were significantly less potent to GABA, a trend we observed with the binary but not the ternary

combinations. This difference may be due to the expression system used and to the type of γ2-subunit used. The γ2-subunit exists in two splice variants, termed γ2S and γ2L. The long-splice variant (γ2L) differs from the short variant (γ2S) by the inclusion of eight additional amino acids in the intracellular loop between TM3 and TM4. In this study, we used the γ2L-subunit, while Mortensen and colleagues used the γ2S-subunit. The variability may be related to a difference in phosphorylation. The additional amino acids within the TM3 and TM4 loop contain an extra phosphorylation site, which may under certain conditions influence the GABA potency (Brandon et al. 2000).

At γ2L-containing receptors, the β2- or β3-subunits did not have a major impact on GABA potency. However, in β1-containing receptors, GABA potency varied in a similar manner to their binary counterparts, i.e., GABA did not significantly differ among the α2-, α4–6-subunits, while the least sensitive receptors to GABA were the α1- and α3-containing receptors.

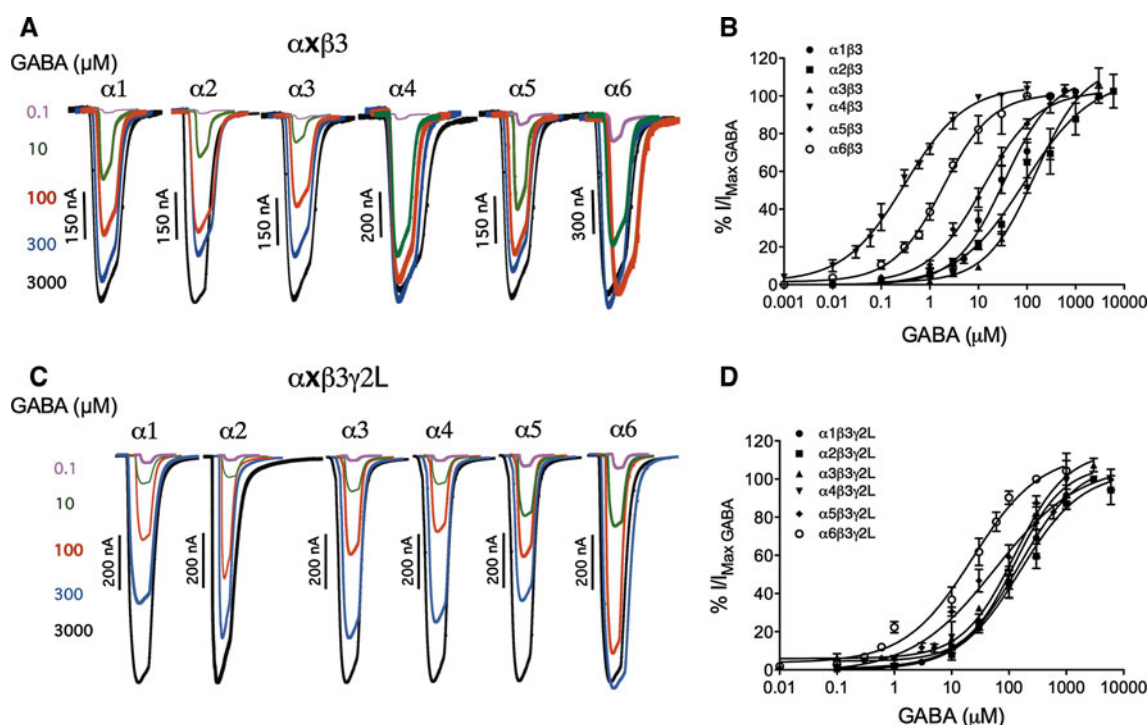The γ2L-subunit did not affect GABA potency, as there is no GABA binding site located on this subunit. In

Fig. 4 Assessing the impact of α-subunits and β3-subunit alone, and with γ2L-subunit on GABA potency. **a** Superimposed membrane currents activated by GABA concentrations ranging from 0.1 μM to 3 mM for α1–6β3 GABA$_A$ receptors expressed in *Xenopus* oocytes. **b** Dose–response curves for GABA at α1β3 (*filled circle*) α2β3 (*filled square*) α3β3 (*filled upward triangle*) α4β3 (*filled downward triangle*) α5β3 (*filled diamond*) and α6β3 (*open circle*) receptor subtypes expressed in *Xenopus* oocytes. **c** Superimposed membrane currents activated by GABA concentrations ranging from 0.1 μM to 3 mM for α1–6β3γ2L GABA$_A$ receptors expressed in *Xenopus* oocytes. **d** Dose–response curves for GABA at α1β3γ2L (*filled circle*), α2β3γ2L (*filled square*), α3β3γ2L (*filled upward triangle*), α4β3γ2L (*filled downward triangle*), α5β3γ2L (*filled diamond*) and α6β3γ2L (*open circle*) receptor subtypes expressed in *Xenopus* oocytes. Data are expressed as mean ± SEM ($n = 3$–10)

contrast, the δ-subunit had a major impact on GABA potency. δ-Containing GABA$_A$ receptors were highly sensitive to GABA, activating α4βδ and α6βδ GABA$_A$ subtypes in the nanomolar range (Karim et al. 2012). The potency for GABA was also highly influenced by the type of β-subunit. When β1- and β3-subunits are combined with α4/δ-subunits, GABA displayed low nanomolar potency, while at the α4β2δ combination, GABA had micromolar potency. In contrast, GABA had low nanomolar activity with the α6β2δ, while activated α6β1δ and α6β3δ with high nanomolar concentrations.

Interestingly, at α6β2δ and α4β3δ GABA$_A$ subtypes, the effects of GABA preferentially fitted a two-site model. This indicates that mixed populations of receptors are being formed when the oocyte system is used (Hadley and Amin 2007; Karim et al. 2012). Varying the ratio of cRNA injected into the oocyte, an approach extensively used with nACh receptors, varied the population of receptors being expressed (Hadley and Amin 2007; Karim et al. 2012). Whether these receptors exist as different stoichiometric forms as evidenced from concatenated receptors (Kaur et al. 2009; Sigel et al. 2009) or whether there is "contamination" of α4β3 receptors (Meera et al. 2011; Karim et al. 2012), as evidenced by Zn^{2+}

Table 3 Effect of GABA at δ-subunit containing GABA$_A$ receptors (Karim et al. 2012)

Receptor subtype	GABA EC$_{50}$ (μM) (95 % CI)	n_H	n
α1β3δ	8.7 (6.4–11.7)	0.8 ± 0.3	4
α4β1δ	0.024 (0.019–0.030)	1.1 ± 0.1	8
α4β2δ	1.00 (0.89–1.31)	1.3 ± 0.2	4
α4β3δ biphasic 5:1:5 injection ratio	EC$_{50}$ (1) = 0.012 (0.006–0.025)	n_H (1) = 1.2 ± 0.5	24
	EC$_{50}$ (2) = 1.3 (0.7–2.5)	n_H (2) = 1.1 ± 0.3	
α4β3δ monophasic	0.016 (0.014–0.018)	0.8 ± 0.1	5
α6β1δ	0.35 (0.24–0.52)	0.9 ± 0.3	6
α6β2δ	EC$_{50}$ (1) = 0.05 (0.02–0.15)	n_H (1) = 0.9 ± 0.3	4
	EC$_{50}$ (2) = 8.7 (3.7–20)	n_H (2) = 1.7 ± 1.0	
α6β3δ	0.44 (0.33–0.53)	0.9 ± 0.4	10

inhibition of higher GABA concentrations is yet to be determined. Interestingly, when using HEK cells, only the low affinity α4β3δ receptors are expressed.
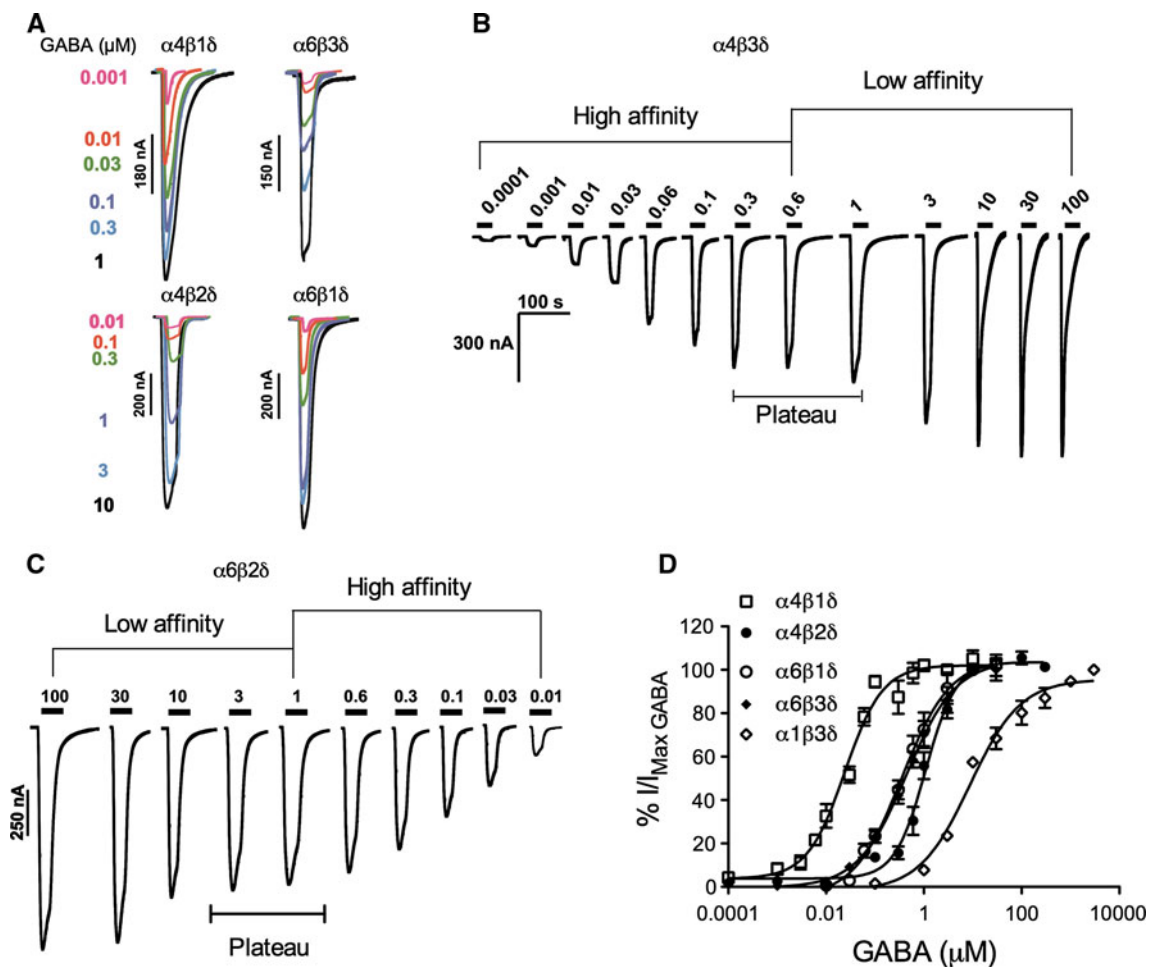
Fig. 5 Assessing the impact of δ GABA$_A$ receptors subunit on GABA potency. Representative traces showing membrane currents activated by various GABA concentrations at **a** α4β1δ, α6β3δ, α4β2δ and α6β1δ GABA$_A$ receptors expressed in *Xenopus* oocytes. **b, c** Representative traces for showing membrane currents activated by various GABA concentrations. Traces highlight the high and low affinity components and the plateau response representative of a bi-phasic concentration response curves at α4β3δ and α6β2δ GABA$_A$ receptors expressed in *Xenopus* oocytes. **d** Concentration–response curves for GABA at α4β1δ (*open square, n* = 8), α4β2δ (*filled circle, n* = 4), α6β1δ (*open circle, n* = 6), α6β3δ (*filled diamond, n* = 10), and compared to α1β3δ (*open diamond, n* = 4) GABA$_A$ receptor subtypes. Data are expressed as mean ± SEM

Given the high sequence identity of the GABA binding site, it is not clear why GABA is so potent at α4β3δ GABA$_A$ receptors. One possibility may be that the δ-subunit is not just a substitute for the γ2-subunit, but may contain an additional GABA binding site. Using the α4β3δ GABA$_A$ receptor, Karim et al. (2012) found that GABA potency was affected not only by the β3-subunit but, to some extent, by the δ-subunit. Furthermore, mutations in the β3- but not α4-subunit, known to affect GABA potency on analogous synaptic α1β2γ2L GABA$_A$ receptors, had little effect on GABA potency at α4β3 and α4β3δ (Karim et al. 2012) and, α4β1 and α4β1δ GABA$_A$ receptors (Absalom et al. 2012), inferring that the binding mode of GABA is different for this receptor. In addition, a mutation in loop C of the δ-subunit affected GABA potency possibly indicating an additional binding site, which may explain its potency on these receptors (Karim et al. 2012).

In conclusion, it is not surprising that differences arise in GABA potency when evaluated using the various expression systems (oocytes versus HEK293 cells for example). This most likely arises come from the amount of cRNA or cDNA that is injected or transfected, but other factors may also play a role (such as phosphorylation state, differences related to the drug application system, the size of the cell, and the presence of endogenous factors in oocytes). In the case of oocytes, the amount of cRNA injected is well controlled. Indeed varying the relative amount of mRNA injected affects what GABA$_A$ receptor subtypes are expressed (Boileau et al. 2002). Boileau et al. (2002) show that mixed populations of binary and ternary receptors are

formed if the ratios are not optimal. In our laboratory, we inject oocytes with excess γ2- or δ-subunit mRNAs (either as 1:1:5, 1:1:10 or 5:1:5 for the α4β3δ combination) to avoid contamination by binary receptors that will lower the overall EC_{50} value. In addition, we used Zn^{2+} to ascertain that ternary combinations express as a homogeneous population. However, the slow application speed when using oocytes may desensitize the receptor, increasing the overall EC_{50} value. With the exception of δ-containing $GABA_A$ receptors, the EC_{50} values obtained using oocytes were indeed higher (approx. 4 to 10-fold) compared to those obtained from HEK293 cells (Mortensen et al. 2012), and may reflect the slow application speed.

In summary, differences in GABA potency at the various receptors may reflect that different GABA concentrations are required to activate $GABA_A$ receptors located either synaptically or extrasynaptically. Given that α1–3-containing receptors located on postsynaptic sites, exposure to high concentrations of GABA is required for activation, while at extrasynaptic sites, receptors composed of α4–6 require less GABA for activation (Mortensen et al. 2012). Our data reflect this trend and the vast number of human $GABA_A$ receptor subtypes studied will provide a basis for comparing the effect of GABA using the *Xenopus* oocyte system in one laboratory.

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