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The distinctive assembly pattern of ε subunit in ternary α1β3ε and binary β3ε GABA_A receptors

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Abstract

Among all GABA_A receptor subunits, ε subunit is a more recent discovery. ε subunit-containing GABA_A receptors exhibit spontaneous channel activity, rapid desensitisation, low sensitivity to Zn^{2+}, smaller GABA-mediated current amplitudes and an insensitivity to benzodiazepines, despite displaying an agonistic effect at higher benzodiazepine concentrations. The promiscuous role of the ε subunit, co-assembling with other subunits forming GABA_ARs, may add to the complexities of the pharmacological properties of GABA_ARs; however, these varying pharmacological responses can be used to distinguish varying subunit combinations of these receptors.

Using two electrode voltage-clamped electrophysiology, we investigated the GABA_A α1β3ε and β3ε receptors and explored the effects of different stoichiometries of these receptor subtypes by varying the relative ratios of α1/β3/ε (for α1β3ε receptors) and β3/ε (for β3ε receptors) subunit complementary RNA injections into Xenopus laevis oocytes. We discovered the existence of different populations of GABA_A α1β3ε and β3ε receptors, due to subunit ratio variation, in which receptors formed at each injection ratio showed different level of GABA sensitivities, spontaneous current activities and Zn^{2+}-mediated current inhibition. These unique pharmacological features are tightly associated with various subtypes of GABA_A receptors contributed by the unique assembly pattern of ε subunit.

Keywords: GABA_A receptors, ε subunit, subtype, stoichiometry

Introduction

Gamma-aminobutyric acid type A receptors (GABA_ARs) mediate the majority of the rapid inhibitory transmissions that occur in the adult central nervous system (CNS) (Farrant and Nusser, 2005; Farrant and...
subunits that can assemble into GABA ARs obey strict rules that define the specificity of the receptor subtype formed (Miller and Aricescu, 2015; Sigel and Steinmann, 2012). This subunit diversity has been suggested to be tightly associated with the extensive heterogeneity observed among receptor subtypes, which determines the pharmacological nature of the receptor and how it responds to agonists, antagonists, and receptor modulators (Farrant and Kaila, 2007; Olsen and Sieghart, 2009). Because different receptor subtypes are distributed in distinct cellular and subcellular locations, which could influence their pharmacological properties and neuron-specific functions, the characterisation of the pharmacological and biophysical features of each receptor subtype is necessary.

The most abundant GABA_A subtype in the brain is stoichiometrically composed of two α1, two β2 and one γ2 subunit, arranged in the anti-clockwise configuration of γ2β2α1β2γ2 when viewed from synaptic cleft (Baumann et al., 2002; Farrant and Nusser, 2005; Farrant and Kaila, 2007; Sigel and Steinmann, 2012; Mortensen et al., 2012). Major subunits, such as α1, β1, β2, β3 and γ2, are widely distributed throughout the brain, although differences in their distribution patterns have been observed (Pirker et al., 2000; Olsen and Sieghart, 2009). However, the distributions of the α2, α3, α4, α5, α6, γ1, δ and θ subunits appear to be restricted to certain brain regions (Olsen and Sieghart, 2009; Pirker et al., 2000; Uusi-oukari and Korpi, 2010). In contrast, the majority of the ρ1-3 subunits are localised in the retina, superior colliculus and cerebellum (Boue-grabot et al., 1998; Wegelius et al., 1998; Uusi-oukari and Korpi, 2010). Meanwhile, the π subunit has not been detected in the brain and, instead, is primarily abundant in the uterus (Hedblom and Kirkness, 1997; Uusi-oukari and Korpi, 2010). Despite those informative findings, little is known regarding the ε subunit, and the pharmacological features of this subunit remain elusive and controversial. In 1997, the ε subunit was independently cloned and functionally characterised (Davies et al., 1997; Whiting et al., 1997). The ε subunit shares 38-47% amino acid homology with the γ subunits, 28-30% homology with α subunits and less than 25% homology with the other GABA_A subunits (Davies et al., 1997; Bollan et al., 2008). In fact, the maximum levels of sequence homology are similar to those that have been reported between the different classes of other GABA_AR subunits, supporting the categorisation of the ε subunit within a different subunit class (Davies et al., 1997).

In the brain, the ε subunit is primarily enriched in brain regions that include the amygdala, the hypothalamus, the subthalamic region and the locus coeruleus, where this subunit forms a cluster with the α3 subunit gene in chromosome Xq28 (Davies et al., 1997; Whiting et al., 1997; Korpi et al., 2002). In contrast with most other GABA_AR subunits, the incorporation of the ε subunit within the receptor complex results in receptors that possess unique biophysical and pharmacological features, including spontaneous channel activity (agonist-independent) that is sensitive to picrotoxin (Neelands et al., 1999). In addition, it has also been discovered that ε subunit-containing receptors demonstrate rapid receptor desensitisation, low sensitivity to Zn^{2+}, smaller GABA-mediated current amplitudes and an insensitivity to low concentrations of benzodiazepines, despite displaying an antagonistic effect at higher (micromolar) benzodiazepine concentrations (Whiting et al., 1997; Davies et al., 1997; Neelands et al., 1999; Thompson et al., 1998; Davies et al., 2001; Thompson et al., 2002; Maksay et al., 2003; Jones et al., 2006; Wagner et al., 2005). Additionally, the ε subunit-containing receptors have also been shown to be insensitive to the potentiating effects of anaesthetics and neurosteroids (Whiting et al., 1997; Davies et al., 1997; Thompson et al., 1998; Davies et al., 2001; Thompson et al., 2002). However, it was reported that these receptors are only insensitive to the anaesthetic-mediated potentiating effects while retaining their sensitivity to the activating effects of the anaesthetic agent, suggesting that distinct anaesthetic binding sites may mediate the two different responses (Sanna et al., 1995; Davies et al., 1997; Thompson et al., 2002). Contrastingly, some
studies have indicated that these receptors did not confer insensitivity to anaesthetics but were instead directly activated or potentiated by pentobarbital, anaesthetics and neurosteroids (Whiting et al., 1997; Neelands et al., 1999).

In the ternary $\alpha\beta\gamma$ receptors, it has been proposed that the $\varepsilon$ subunit may substitute the $\gamma$ position, forming an $\alpha\beta\varepsilon$ receptor (Jones and Henderson, 2007). These inconsistent findings, particularly with regard to the pharmacology of anaesthetics, may be due to the formation of heterogeneous receptor populations, caused by differences in $\varepsilon$ subunit expression levels. These discrepancies have been suggested to arise from inconsistencies in the expression levels of the $\varepsilon$ subunit relative to those for the $\alpha$ and $\beta$ subunits, which could result in the formation of other receptor combinations, in addition to the expected $2\alpha:2\beta:1\varepsilon$ receptor stoichiometry (Thompson et al., 2002). The presence of differing receptor populations, due to variations in the $\varepsilon$ subunit-containing receptor stoichiometry, particularly for the ternary $\alpha\beta\varepsilon$ GABA$_A$Rs, could influence the pharmacological receptor properties, including the potentiating effects triggered by anaesthetics, the levels of spontaneous channel currents, and the sensitivity to GABA (Thompson et al., 2002). In addition, an in vitro study discovered that the ternary $\alpha1\beta3\varepsilon$ GABA$_A$R subtype formed functional chloride ion channels that were both spontaneously active and gated by GABA (Neelands et al., 1999). However, adding further complexity, it has been reported that the $\varepsilon$ subunit may not only replace the $\gamma$ subunit in ternary $\alpha\beta\gamma$ receptors but may also replace either of the $\alpha$ or $\beta$ subunits to form stoichiometrically and functionally distinct $\alpha\beta\varepsilon$ receptors, further validating the promiscuous ability of the $\varepsilon$ subunit to assemble with other subunits (Davies et al., 2001; Jones and Henderson, 2007; Bollan et al., 2008).

Therefore, in this study, we aimed to further characterise the pharmacological properties of GABA$_A\alpha1\beta3\varepsilon$ receptors and to explore the effects of different stoichiometries this receptor subtype by varying the relative ratios of $\alpha1$, $\beta3$ and $\varepsilon$ subunit complementary RNA (cRNA) injections into Xenopus laevis oocytes. In addition, we characterised and distinguished the expressed receptors resulting from different injection ratios by their distinct pharmacological responses to GABA and Zn$^{2+}$, based on two-electrode voltage clamp electrophysiology recordings.

Materials and Methods

Chemicals

GABA ($\gamma$-aminobutyric acid), sodium pyruvate, theophylline, gentamycin and zinc chloride were obtained from Sigma Aldrich, USA. Tricaine was purchased from Western Chemical, USA.

Human GABA$_A$R subunit cDNA

Human GABA$_A$R subunit cDNAs, including $\alpha1$ subcloned into the pcDM8 vector, $\beta3$ subcloned into the pGEMHE vector and $\varepsilon$ subcloned into the pcMV6 vector, were linearized with the appropriate restriction endonucleases (NotI for $\alpha1$, NheI for $\beta3$, and SmaI for $\varepsilon$). Concatenated $\beta3$-$\alpha1$ subunits were developed, as previously described (Baumann et al., 2001), subcloned into the pNS3z vector, and linearized with NotI. cRNA was produced from the linearized plasmids using the ‘mMessage mMachine’ T7 transcript kit from Ambion (Austin, TX, USA). Up to 50 nl, containing 5-8 ng of cRNA, was injected per oocyte. When using free subunits of $\alpha1$, $\beta3$ and $\varepsilon$, the cRNAs were mixed in two different $\alpha1:\beta3:\varepsilon$ ratios; 1:1:3 and 10:1:30. To ensure the incorporation of a free $\varepsilon$ subunit in the pentameric complex, concatenated $\beta3$-$\alpha1$ cRNA was injected with $\varepsilon$ cRNA at a 1:2 ratio. Free $\beta3$ and $\varepsilon$ subunit cRNAs were mixed in two different $\beta3:\varepsilon$ ratios; 1:3 and 1:5, for the formation of binary $\beta3\varepsilon$ receptors.

Preparation of Xenopus oocytes

Procedures using Xenopus laevis frogs were approved by the animal ethics committee of Universiti Sains Malaysia (No. of Animal Ethics Approval: USM/2015/(98)(698)) and are in accordance to The Animal Ethics Committee of Universiti Sains Malaysia. In brief, Xenopus laevis were anaesthetised using a 0.2% w/v solution of tricaine methanesulfonate or tricaine-S (Western Chemical, USA). A transverse incision of approximately 3 mm in length was made through the outer layer of the skin, on the lateral ventral surface. Another incision was made through the connective tissue and muscle layer to reach the ovary wall. A section of the ovary was carefully removed onto the surface of the frog. A small section of ovary was separated and transferred into a tube containing OR2 solution (82.5 mM NaCl, 5 mM HEPES, 2 mM MgCl$_2$ and 2 mM KCl, pH 7.4). Oocytes were separated manually from their follicles and digested with 40 mg collagenase diluted in 15 ml OR2

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solution, at 18°C, for approximately 1 hour, until the oocytes were fully detached from the follicles and the ovary tissue.

Stage V-VI oocytes were injected with 50 nl cRNA solution, composed of GABA$_A$Rs subunit cRNAs in the required ratios. The injected oocytes were incubated in ND96 solution (96 mM NaCl, 5 mM HEPES, 2 mM MgCl$_2$, 1 mM KCl and 1.8 mM CaCl$_2$, pH 7.4) supplemented with 2.5 mM Na-pyruvate, 0.5 mM theophylline, 50 mg/ml gentamycin and 50 mg/ml tetracycline for 2-5 days at 18°C.

Electrophysiology

The electrophysiological experiments were conducted using the two-electrode voltage clamp technique. The membrane potential was measured using an Oocyte Clamp OC-725C amplifier (Warner Instrument Corp, CT, USA), using a voltage electrode combined with the simultaneous injection of current through the current electrode to maintain a potential difference across the oocyte membrane of -60 mV. Data were acquired with a LabChart v. 3.5.2 analogue to digital converter, and currents were low-pass-filtered at 1 kHz and sampled at 3 kHz, then measured offline using LabChart v. 3.5.2 software. The bath solution contained the ND96 solution, and the electrodes were filled with a 3 M KCl solution (0.5-2 MΩ). Solutions were bath-applied using a gravity-fed perfusion system running at 5 ml/min.

For Zn$^{2+}$ inhibition studies, control currents ($I_{\text{control}}$) were evoked using a GABA concentration corresponding to the EC$_{50}$, and the inhibition by Zn$^{2+}$ was evaluated by the co-application of Zn$^{2+}$ with GABA$_{\text{control}}$. The averaged Zn$^{2+}$ inhibition in the presence of GABA at EC$_{50}$ concentrations were depicted as the mean ± S.E.M, as a function of the Zn$^{2+}$ concentration, and fitted to the Hill equation by non-linear regression. The enhancement of GABA-gated Cl$^-$ currents was measured by co-applying modulators with a GABA concentration that elicited 5% of the maximal current amplitude, as determined at the beginning of each experiment. The enhancement of the GABA-gated Cl$^-$ current ($I_{\text{GABA}}$) was defined as $I = I_{\text{max}}/(1 + [\text{EC}_{50}(A)]^{nH})$; where A is the agonist concentration, I is the current, $I_{\text{max}}$ is the maximum current, EC$_{50}$ is the concentration of GABA that produces a response that is 50% of the maximum current, and $n_H$ is the Hill Coefficient. The EC$_{50}$ value is expressed as the mean ± S.E.M.

Concentration-response curves were generated, and the data were fitted by non-linear regression analysis using GraphPad Prism software (version 5.0). Statistical significance was calculated using unpaired Student’s t-tests, with a confidence interval of $p < 0.05$, to compare parameters derived from individual experiments, and data are presented as the mean, with a 95% confidence interval, from at least 5 oocytes and at least 2 different batches. The logEC$_{50}$ values, derived from individual comparisons, were used for statistical comparisons.

Results

Different populations of GABA$_A$ α1β3ε receptors are expressed when the injection ratio of α1/β3/ε cRNA is varied

Up to now, little information has been reported regarding the ε subunit assembly into GABA$_A$Rs (Bollan et al., 2008). Recent studies have suggested that this subunit may not only displace the γ2 subunit but may also displace either of the α or β subunits (Bollan et al., 2008; Davies et al., 2001; Jones and Henderson, 2007). Thus, to stoichiometrically vary the population of the ε-containing GABA$_A$Rs and to favour the displacement of either of the β3 subunits, the following α1/β3/ε cRNA injection ratios were used for injection into Xenopus oocytes: 1:1:3 and 10:1:30. To ensure a reliable comparison between the expressed receptors at each injection ratio, all cRNAs were derived from a single stock solution using the same cRNA concentration per oocyte. The holding currents and GABA sensitivities between receptors formed using the 1:1:3 and 10:1:30 injection ratios were compared.

When the membrane potentials were clamped at -60 mV, the receptors formed by the 1:1:3 and 10:1:30 ratios exhibited holding currents of -400 ± 120 nA (n = 5) and -1600 ± 400 nA (n = 5), respectively (Figure 1 A and B). The expressed receptors from both injection ratios were activated by GABA in a concentration-dependent manner, and the EC$_{50}$ for GABA was significantly higher ($p < 0.0001$, Student’s t-test, log EC$_{50}$) for the receptors formed by the 1:1:3 (3.3 ± 2.0 µM; Table 1, Figure 1C) injection ratio than for those formed by the 10:1:30 injection ratio (0.3 ± 0.13 µM; Table 1, Figure 1C), demonstrating the reduced GABA sensitivity of receptors formed at the 1:1:3 injection ratio compared with those formed at the 10:1:30 injection ratio. This heterogeneity suggests the formation of different receptor
The ε subunit in GABA\textsubscript{A} receptors stoichiometries and supports the use of varying the cRNA injection ratio to vary the receptor subunit stoichiometry.

It was initially suggested that the ε subunit may simply replace the γ2 subunit within the receptor complex (Neelands et al., 1999). To ascertain whether this occurs, we investigated the existence of heterogeneities among the pharmacological properties of the receptors formed by the co-expression of concatenated β3-α1 subunits and the ε subunit, (β3-α1) + ε and of the receptors formed using a 1:1:3 α1/β3/ε injection ratio. In this study, we constructed the concatenated β3-α1 subunits by linking the N-terminal of the α1 subunit to the C-terminal of the β3 subunit. The concatenated (β3-α1) + ε condition was used to stoichiometrically restrict the receptor complex formation to 2α1:2β3:1ε. Holding currents of −400 ± 75 nA (n = 5) were recorded from the concatenated (β3-α1) + ε receptors when the membrane potentials were clamped at −60 mV (Figure 2A). When the GABA concentration-response curves for both the (β3-α1) + ε and the 1:1:3 α1/β3/ε receptors were overlaid, they exhibited homologous curves and non-significant (p > 0.05, Student’s t-test, logEC\textsubscript{50}) GABA sensitivity, with EC\textsubscript{50} values of 1.6 ± 0.9 µM for (β3-α1) + ε and 3.3 ± 2.0 µM for 1:1:3 (Figure 2B, Table 1). The homogeneity between the concatenated (β3-α1) + ε and the 1:1:3 injection ratio conditions demonstrates that this ratio results in the formation of receptors with the native 2α1:2β3:1ε stoichiometry.

Table 1. GABA concentration response relationships at various GABA\textsubscript{A} receptors. Xenopus laevis oocytes were injected with cRNA mixtures containing the indicated GABA\textsubscript{A} receptor subunits. Background subtracted peak current amplitudes for full GABA concentration-response curves in presence or absence of zolpidem were fitted to the Hill equation (fixed bottom of 0 and slope of 1) using non-linear regression and normalized to the maximal fitted value. Averaged normalized data points were next fitted to the Hill equation and resultant EC\textsubscript{50} values and maximal efficacies (E\textsubscript{max}) are presented as mean with 95% confidence intervals for n experiments.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>cRNA ratio</th>
<th>EC\textsubscript{50} (µM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1 + β3 + ε</td>
<td>1:1:3</td>
<td>3.3 ± 2.0</td>
<td>5</td>
</tr>
<tr>
<td>α1 + β3 + ε</td>
<td>10:1:30</td>
<td>3.3 ± 0.13</td>
<td>5</td>
</tr>
<tr>
<td>(β3-α1) + ε</td>
<td>1:2</td>
<td>1.6 ± 0.9</td>
<td>5</td>
</tr>
<tr>
<td>β3 + ε</td>
<td>1:3</td>
<td>26 ± 2.7</td>
<td>5</td>
</tr>
<tr>
<td>β3 + ε</td>
<td>1:5</td>
<td>2.3 ± 1.4</td>
<td>5</td>
</tr>
</tbody>
</table>

Figure 1. GABA-evoked responses at α1β3ε GABA\textsubscript{A} receptors under different ratios; 10:1:30 and 1:1:3. Xenopus laevis oocytes were injected with cRNA and subjected to two-electrode voltage-clamp electrophysiology as described in the methods. For experimentation, oocytes were clamped at −60 mV and full GABA concentration response relationships were obtained on each oocyte. (A, B) Representative GABA-evoked traces from oocytes injected with the denoted cRNA mixtures. Dotted lines indicate holding currents at −1600 ± 400 nA, n = 5 for α1β3ε (10:1:30; A) and −400 ± 120 nA, n = 5 for α1β3ε (1:1:3; B). (C) Baseline subtracted peak current amplitudes for full GABA concentration-response curves at oocytes injected with the indicated cRNA mixtures using free subunits and were fitted to the Hill equation using non-linear regression (fixed bottom of 0 and slope of 1) and normalized to the maximal fitted value (I\textsubscript{GABA, max, β3}). Averaged normalized data points are depicted as means ± S.E.M as a function of the GABA concentration, fitted to the Hill equation and regression results are presented in Table 1. Each data point represents experiments from n = 5 oocytes from ≥ 2 batches.

The receptors formed by the co-expression of concatenated β3-α1 subunits and the ε subunit mimic the receptors formed by the 1:1:3 α1:β3:ε injection ratio.

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The ε subunit in GABA<sub>A</sub> receptors

When GABA, at an EC<sub>50</sub> concentration, was co-administered with Zn<sup>2+</sup> (10 µM), the receptors expressed by the 1:1:3 injection ratio exhibited significantly reduced Zn<sup>2+</sup>-induced current inhibition (21 ± 9%, n = 5; Figure 3B) than the receptors expressed by the 10:1:30 injection ratio (57 ± 16%, n = 8; Figure 3B)). The Zn<sup>2+</sup> IC<sub>50</sub> was significantly higher for receptors expressed by the 1:1:3 injection ratio (426 µM, n = 5) than for the receptors expressed by the 10:1:30 injection ratio (8.3 µM, n = 8; Figure 3B), denoting a reduced Zn<sup>2+</sup> sensitivity for the receptors expressed by the 1:1:3 injection ratio compared with the receptors expressed by the 10:1:30 injection ratio. Additionally, for the receptors expressed by the 10:1:30 injection ratio, the GABA-mediated current was inhibited by Zn<sup>2+</sup> at a low ZnCl<sub>2</sub> concentration (0.1 µM) and was almost completely abolished at a high ZnCl<sub>2</sub> concentration (100 µM), whereas for receptors expressed by the 1:1:3 injection ratio, no current inhibition or abolishment were observed at either low or high ZnCl<sub>2</sub> concentrations (Figure 3B).

Effects of Zn<sup>2+</sup> on receptors expressed at different α1/β3/ε ratios

The incorporation of the ε subunit into a ternary GABA<sub>A</sub>Rs complex has been shown to exhibit a lower potency for Zn<sup>2+</sup> compared with binary αβ receptors (Whiting et al., 1997). Therefore, to investigate whether ε subunit-containing GABA<sub>A</sub>Rs can be distinguished by their response to Zn<sup>2+</sup>, we tested the receptor populations from both injection ratios (1:1:3 and 10:1:30) using Zn<sup>2+</sup>.

Figure 2. GABA-evoked responses at concatenated β3-α1 + ε and α1β3ε GABA<sub>A</sub> receptors under 1:1:3 injection ratio. Xenopus laevis oocytes were injected with cRNA and subjected to two-electrode voltage-clamp electrophysiology as described in the methods. For experimentation, oocytes were clamped at −60 mV and full GABA concentration response relationships were obtained on each oocyte. (A) Representative GABA-evoked traces from oocytes injected with the denoted cRNA mixtures. Dotted lines indicate holding current at −400 ± 120 nA, n = 5 for concatenated (β3-α1) + ε. (B) Baseline subtracted peak current amplitudes for full GABA concentration-response curves at oocytes injected with the indicated cRNA mixtures using free subunits and were fitted to the Hill equation using non-linear regression (fixed bottom of 0 and slope of 1) and normalized to the maximal fitted value (I<sub>GABA_max_fit</sub>). Averaged normalized data points are depicted as means ± S.E.M as a function of the GABA concentration, fitted to the Hill equation and regression results are presented in Table 1. Each data point represents experiments from n = 5 oocytes from ≥ 2 batches. (C) Receptor stoichiometry for α1:β3:ε under 1:1:3 injection ratio arranged at position 1-5 in the anticlockwise pattern (top), and concatenated (β3-α1) + ε (bottom). The two binding sites for GABA at β3(+)-α1(-) subunit interface are indicated by solid red arrowheads. Links between β3 and α subunits forming the concatenated β3-α constructs are depicted as solid black lines.

Figure 3. Zn<sup>2+</sup> inhibition of GABA-evoked currents from concatenated β3-α1 + ε and α1β3ε GABA<sub>A</sub> receptors under different ratios; 1:1:3 and 10:1:30. Xenopus laevis oocytes were injected with cRNA and subjected to two-electrode voltage-clamp electrophysiology as described in the methods. For experimentation, oocytes were clamped at −60 mV and full
GABA concentration response relationships were obtained on each oocyte. (A) Averaged Zn$^{2+}$ inhibition values were depicted as means ± S.E.M as a function of the Zn$^{2+}$ concentration and fitted to the Hill equation by non-linear regression. Regression results for α1:β3:ε (1:1:3) were IC$_{50}$ = 426 (µM) and for concatenated β3-α1 + ε were IC$_{50}$ = 1.6 µM (95% CI: 1.1–2.4). For the remaining cRNA mixtures, Zn$^{2+}$ inhibition at the maximal tested concentration was too low to allow for meaningful fitting. Each data point represents experiments from n = 5 – 8 oocytes of ≥ 2 batches.

(B) Averaged Zn$^{2+}$ inhibition values were depicted as means ± S.E.M as a function of the Zn$^{2+}$ concentration and fitted to the Hill equation by non-linear regression. Regression results for α1:β3:ε (1:1:3) were IC$_{50}$ = 426 µM and for α1:β3:ε (10:1:30) were IC$_{50}$ = 8.3 µM. For the remaining cRNA mixtures, Zn$^{2+}$ inhibition at the maximal tested concentration was too low to allow for meaningful fitting. Each data point represents experiments from n = 5 - 8 oocytes of ≥ 2 batches.

Then, we compared the effects of Zn$^{2+}$ on the expressed receptors expressed from both the 1:1:3 injection ratio and the concatenated (β3-α1) + ε conditions (Figure 3A). Zn$^{2+}$ (10 µM) inhibited 16 ± 5% (n = 5) of the current elicited by the GABA EC$_{50}$ concentration in the concatenated (β3-α1) + ε condition (Figure 3A) and 21 ± 9% (n = 5) of the current in the 1:1:3 injection ratio condition. Based on the Zn$^{2+}$ concentration-inhibition curves (Figure 3A), the Zn$^{2+}$ sensitivity of the receptors expressed under the concatenated (β3-α1) + ε condition (n = 5) was significantly different from that of the receptors expressed under the 1:1:3 injection ratio condition (p < 0.001, Student’s t-test, n = 5).

Unique pharmacological properties of binary β3ε GABAA receptors

Neelands and colleagues have shown the existence of binary β3ε GABAA receptors, which were activated by pentobarbital, despite being insensitive to GABA, and failed to display significant spontaneous current activity (Neelands et al., 1999). To validate this evidence, we investigated whether binary β3ε receptors expressed at two different injection ratios, 1:3 and 1:5, demonstrated different levels of sensitivity to various concentrations of GABA (100 nM to 1 mM, Figure 4A and 4B) and exhibited any spontaneous current activity. Additionally, to further confirm that both injection ratios result in the formation of binary β3ε receptors, instead of homomeric β3 receptors, we compared the concentration-response curves between the two injection ratios (Figure 4C).

The GABA EC$_{50}$ of the β3ε receptors expressed at the 1:3 injection ratio was significantly higher (48 µM, n = 5) than that of the β3ε receptors expressed at the 1:5 injection ratio (1.5 µM, n = 5), demonstrating reduced GABA sensitivity for the receptors expressed at the 1:3 injection ratio compared with those expressed at the 1:5 injection ratio. Moreover, none of the receptor populations expressed at either injection ratios exhibited significant spontaneous current activity. However, the β3ε receptor populations for both injection ratios showed distinct concentration-response curves from that for homomeric β3 (Figure 4C), indicating that the receptors formed from both injection ratios are binary β3ε receptors instead of homomeric β3 receptors.
the Hill equation using non-linear regression (fixed bottom of 0 and slope of 1) and normalized to the maximal fitted value ($I_{GABA, max,fit}$). Averaged normalized data points are depicted as means ± S.E.M as a function of the GABA concentration, fitted to Hill equation. Each data point represents experiments from $n = 5$ oocytes from ≥ 2 batches.

**Discussion**

Relative to the other GABA$_A$R subunits, the ε subunit is a more recent discovery. Therefore, less information is known regarding the contributions of the ε subunit to the biophysical and pharmacological responses of the receptors, and little is known regarding how it assembles with other subunits, such as with the α and β subunits (Davies et al., 1997; Bollan et al., 2008). We decided to investigate GABA$_A$ α1β3ε receptors because this subtype has been reported as the first ternary receptor to have both GABA-activated and spontaneous channel activities (Neelands et al., 1999; Maksay et al., 2003). The amino acid sequence of the assembly domain of the ε subunit is highly homogenous to that of the β subunits (Jones and Henderson, 2007). This domain is highly crucial for inter-subunit interactions. Therefore, the ε subunit may be able to replace the β subunit from its general position, superseding the vital role played by β3 during receptor formation. The aims of this study were to assess and evaluate the different subunit combinations of GABA$_A$ α1β3ε receptors by varying the injection ratios of α1/β3/ε cRNA. The chosen injection ratios were: 1:1:3 and 10:1:30 α1/β3/ε. The relative concentrations of the β3 subunit differed by 10-fold between the 1:1:3 and 10:1:30 injection ratios. Reducing the amount of β3 cRNA that is injected into Xenopus oocytes by 10-fold might favour the replacement of at least one β3 subunit by the ε subunit, is in addition to retaining an ε subunit at position 5.

In this study, we also attempted to identify the possible receptor stoichiometries by manipulating the α1/β3/ε injection ratio. The heterogeneity of the expressed receptors, due to the variety of injection ratios, were functionally characterised by their distinctive biophysical features. However, the specific receptor subunit configurations remain undetermined. One method for to predetermined the subunit configuration is the use of concatenated GABA$_A$R subunits, known as concatemers. This technique limits the vast possibilities of receptor stoichiometry and subunit configurations to the formation of receptors that contain a single ε subunit (when the β3-

α1 concatemers were co-expressed with the individual ε subunit), with limited subunit arrangement combinations. In this study, the GABA potencies, spontaneous channel activities and current amplitudes observed for the (β3-α1) + ε receptors were observed to be homogenous with those for the receptors expressed by the 1:1:3 α1/β3/ε injection ratio. This suggests that the receptors formed by the 1:1:3 injection ratio most likely contain only a single ε subunit, which further suggests the high feasibility of the ε subunit replacing the γ subunit at position 5 within the GABA$_A$ αβγ receptor complex. However, this finding does not confirm the subunit configuration that results from this injection ratio or whether the configuration is similar to that formed by β3-α1 + ε. The homogenous pharmacological properties observed between the receptors formed by the concatamer and the 1:1:3 α1/β3/ε injection ratio suggests that there is consistency, in terms of receptor functionality and stoichiometry, among the expressed receptors, which may both plausibly be composed of 2α1:2β3:1ε subunits. In addition, in this study, we proposed an alternative and novel technique for studying the pharmacological properties of ε subunit-containing receptors by varying the receptor subunit cRNA injection ratios. Based on our findings, we postulate that the receptors expressed using different injection ratios exhibit heterogeneous pharmacological features, in an ε subunit concentration-dependent manner, which can be distinguished by their responses to GABA and Zn$^{2+}$.

Our data indicate that, using the 10:1:30 injection ratio of α1/β3/ε, the GABA sensitivity of the expressed receptors was higher than that for the expressed receptors using the 1:1:3 injection ratio. At the 10:1:30 injection ratio, the relative reduced concentration of the β3 subunit may explain the presence of an extra ε subunit within the receptor pentamer (likely replacing the β3 subunit), which would affect the subunit configuration. This result further suggests that the replacement of the β3 subunit with an ε subunit or an altered subunit configuration may mediate the receptor sensitivity to GABA. However, it is not possible for the ε subunit to replace all of the β3 subunits in a receptor because at least one β3 subunit must be conserved to form a functional receptor (Bollan et al., 2008; Minier et al., 2004). In fact, the replacement of a β3 subunit with an ε subunit may reduce the available GABA binding sites, thereby decreasing the receptor occupancy for GABA. However, a conserved β3 subunit within the
receptor complex may retain at least one β3+/α1-interface, preserving at least one GABA binding site, which is essential for receptor functionality. The ε subunit-containing GABA_A receptors have been demonstrated to exhibit spontaneous channel activity (Neelands et al., 1999). However, prior studies of native neurons that were thought to express the ε subunit also reported conflicting data regarding the existence of spontaneous GABA_A-mediated currents (Jones et al., 2006; Jones and Henderson, 2007; McDonald et al., 1998; Kasparov et al., 2001; Irmaten et al., 2002; Jorge et al., 2002). Here, we demonstrate that the receptors expressed at each injection ratio displayed distinct spontaneous channel activity. The highest spontaneous channel activity was recorded from the α1/β3/ε injection ratio of 10:1:30, which may be due to the 10-fold difference in the β3 subunit concentration between the 10:1:30 and 1:1:3 injection ratios. A lower β3 subunit concentration could feasibly permit the substitution of a β3 subunit with an ε subunit, which may confer increased spontaneous channel activity.

Furthermore, this result suggests that the presence of the ε subunit, and possibly the presence of an additional ε subunit within an individual receptor complex, may contribute to the high spontaneous current value of the channel. Although the exhibition of spontaneous activities is believed to be ε subunit-dependent, this finding does not clarify the specific ε subunit configurations within the receptor complexes, which likely determine the pharmacological feature(s) of the receptor. Therefore, the position of each subunit remains to be determined. Inconsistencies among the previous findings with regard to spontaneous channel activities in native neurons may be the result of variable configurational arrangements of the ε subunits within the GABA_A receptors. In addition, GABA activation of the receptors expressed by the 10:1:30 injection ratio appeared to result in significantly decreased current amplitudes when compared with those for receptors expressed by the 1:1:3 injection ratio, which is consistent with previous findings (Davies et al., 2001; Sergeeva et al., 2005). This result further corroborates the role played by the ε subunit, either alone or with its α1 and β3 counterparts, in the mediation of reduced GABA-mediated current amplitudes.

The inhibition of ε subunit-containing GABA_A receptors by ZnCl_2 has been demonstrated in a number of studies (Neelands et al., 1999; Davies et al., 2001). Previously, Zn^{2+} has been found to mediate its inhibitory effects on the GABA_A α1β3 receptors via the allosteric site, and the effects of Zn^{2+} have been shown to be crucially dependent on the composition of the α1 and β3 subunits. A molecular modeling study has demonstrated that there are three different sites that mediate Zn^{2+} inhibition (Hosie et al., 2003). The first site is located within the channel, and the other two are located at the extracellular N-terminal interface between the α1 and β3 subunits (Hosie et al., 2003). In α1β3γ2 receptors, the loss of Zn^{2+} inhibition could be due to the presence of the γ2 subunit within the complex and may be explained by the absence of the amino acid residues E182, H267 and E270, which are only present in the β3 subunit, underlying the role of the β3 subunit in the formation of a Zn^{2+} binding site. In this study, compared with the receptors expressed by the 10:1:30 injection ratio, the receptors expressed by the 1:1:3 injection ratio appeared to be less sensitive to Zn^{2+}, which may be explained by the presence of different receptor populations with heterogeneous stoichiometric specificities, particularly the ε subunit stoichiometry, between the two injection ratios. In our previous study, increased Zn^{2+} sensitivity was observed for the binary α1β3 GABA_A receptors, which contain a β3+/β3-interface (Che Has et al., 2016). Therefore, we postulate that for the binary α1β3 GABA_A receptors (which contain the β3+/β3-interface), the incorporation of an ε subunit within the receptor complex would disrupt the β3+/β3-interface, resulting in reduced sensitivity to Zn^{2+}, while simultaneously preserving the α1+/β3-interface that forms the Zn^{2+} binding site, which would explain why receptors formed by the 1:1:3 injection ratio were less sensitive to Zn^{2+}. Our result showed that Zn^{2+} could significantly abolish the GABA-mediated current for receptors expressed by the 10:1:30 injection ratio, signifying the increased sensitivity of the receptor to Zn^{2+}. This result, however, contradicts our previous finding of a correlation between the interruption of the β3+/β3-interface by the ε subunit and reduced Zn^{2+} inhibition. The observed Zn^{2+}-mediated current abolishment is highly similar to that observed for β3+/β3-interface-containing receptors. The preservation of Zn^{2+}-mediated inhibition for receptors expressed by the 10:1:30 injection ratio, even after at least one β3 subunit is replaced by a ε subunit, suggests that the ε subunit is likely to play a significant role in the formation of a Zn^{2+} binding pocket. The positive correlation between ε subunit-containing receptors and the presence of spontaneous channel current...
activity is independent of the role played by the ε subunit during Zn$^{2+}$ inhibition.

In this study, it was shown that the receptors expressed under the concatenated β3-α1 + ε condition displayed reduced Zn$^{2+}$-mediated current inhibition at high Zn$^{2+}$ concentrations than the receptors expressed under the 1:1:3 α1β3ε injection ratio condition. However, the different Zn$^{2+}$ inhibitory responses may be explained by the variable subunit expression profiles for the two conditions. In the concatemer condition, the invariable presence of the ε subunit conferred a reduced receptor sensitivity to Zn$^{2+}$, thereby impeding Zn$^{2+}$ inhibition. However, this current inhibition pattern was not homogenous with that observed under the 1:1:3 injection ratio condition. This result suggests the possibility that the receptor population under the 1:1:3 injection ratio condition may be dominated by binary α1β3 receptors, containing β3+β3- subunit interfaces within the individual receptor complexes. The presence of this interface may explain the different pattern of Zn$^{2+}$-mediated inhibition observed for receptors under the 1:1:3 injection ratio condition than for the concatemer condition. However, this finding does not negate our finding that the biophysical properties of the α1β3ε receptor at the 1:1:3 injection ratio mimics that observed for the β3-α1 + ε receptor, which suggests that the receptor complexes expressed under both conditions are homogenous in their pharmacology and structural stoichiometry. In addition, variations in subunit configurations may also explain heterogeneities among the pharmacological features of these receptors, particularly with regard to Zn$^{2+}$ inhibition. Accordingly, these results may further suggest that the sensitivity of ε subunit-containing receptors to Zn$^{2+}$ is configuration-dependent, rather than merely stoichiometry-dependent. Hence, the specificity of the ε subunit stoichiometry and configuration may be fundamentally responsible for the distinctive intrinsic features of the receptors.

It has been reported that the ε subunit cannot form functional receptors when expressed either alone or when co-expressed with either α alone or β alone (Whiting et al., 1997; Davies et al., 1997). However, Neelands and colleagues have reported the existence of binary β3ε GABA$_A$Rs that can be activated by high concentrations of pentobarbital, despite being insensitive to GABA and failing to display any significant spontaneous current (Neelands et al., 1999). Consistently, in this study, we found the existence of functional binary β3ε receptors. However, our study showed that the binary receptors confer GABA sensitivity in a concentration-dependent manner, with reduced GABA sensitivity observed for the 1:3 injection ratio than for the 1:5 injection ratio. Accordingly, these differences, particularly with regard to GABA sensitivity, suggest the expression of two distinct receptor populations between the two groups. Therefore, the presence of various ε subunit stoichiometries within the receptor complex, due to different proportions of ε subunit cRNA relative to other subunits, may also explain the observed GABA sensitivity differences between the two injection ratios. The ε subunit stoichiometric specificity may be a key factor in the determination of the pharmacology of the receptor subtype.

In this study, we demonstrated that differences in the pharmacological properties of GABA$_A$Rs α1β3ε subtypes are primarily determined by the unique ability of the ε subunit to position itself in various subunit combinations, which influences the receptor stoichiometry and the subunit configuration of the receptor complex. In our study, the receptors formed at 10:1:30 and 1:1:3 injection ratios of α1/β3/ε subunit cRNAs can be differentiated by their sensitivities to GABA, the presence of spontaneous channel activity and the Zn$^{2+}$ inhibition. However, the specific subunit arrangements of each receptor expressed at the different cRNA injection ratios remain unknown.

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The ε subunit in GABAA receptors