

RESEARCH PAPER

Etomidate produces similar
allosteric modulation in
 $\alpha 1\beta 3\delta$ and $\alpha 1\beta 3\gamma 2L$ GABA_A
receptors

H-J Feng, Y Jounaidi, M Haburcak, X Yang and S A Forman

*Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital and
Harvard Medical School, Boston, MA, USA*

Correspondence

Hua-Jun Feng, Department of
Anesthesia, Critical Care and
Pain Medicine, Massachusetts
General Hospital and Harvard
Medical School, 55 Fruit Street,
Boston, MA 02114, USA. E-mail:
hfeng3@partners.org

Keywords

allosteric modulation; etomidate;
general anaesthetics; GABA_A
receptors; δ subunits;
concatenated subunit assemblies;
electrophysiology

Received

27 June 2013

Revised

13 October 2013

Accepted

31 October 2013

BACKGROUND AND PURPOSE

Neuronal GABA_A receptors are pentameric chloride ion channels, which include synaptic $\alpha\beta\gamma$ and extrasynaptic $\alpha\beta\delta$ isoforms, mediating phasic and tonic inhibition respectively. Although the subunit arrangement of $\alpha\beta\gamma$ receptors is established as $\beta-\alpha-\gamma-\beta-\alpha$, that of $\alpha\beta\delta$ receptors is uncertain and possibly variable. We compared receptors formed from free $\alpha 1$, $\beta 3$ and δ or $\gamma 2L$ subunits and concatenated $\beta 3-\alpha 1-\delta$ and $\beta 3-\alpha 1$ subunit assemblies (placing δ in the established γ position) by investigating the effects of R-(+)-etomidate (ETO), an allosteric modulator that selectively binds to transmembrane interfacial sites between $\beta 3$ and $\alpha 1$.

EXPERIMENTAL APPROACH

GABA-activated receptor-mediated currents in *Xenopus* oocytes were measured electrophysiologically, and ETO-induced allosteric shifts were quantified using an established model.

KEY RESULTS

ETO (3.2 μ M) similarly enhanced maximal GABA (1 mM)-evoked currents in oocytes injected with 5 ng total mRNA and varying subunit ratios, for $\alpha 1\beta 3(1:1)$, $\alpha 1\beta 3\delta(1:1:1)$ and $\alpha 1\beta 3\delta(1:1:3)$, but this potentiation by ETO was significantly greater for $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1(1:1)$ receptors. Reducing the amount of $\alpha 1\beta 3\delta(1:1:3)$ mRNA mixture injected (0.5 ng) increased the modulatory effect of ETO, matching that seen with $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1(1:1, 1 \text{ ng})$. ETO similarly reduced EC₅₀s and enhanced maxima of GABA concentration-response curves for both $\alpha 1\beta 3\delta$ and $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1$ receptors. Allosteric shift parameters derived from these data depended on estimates of maximal GABA efficacy, and the calculated ranges overlap with allosteric shift values for $\alpha 1\beta 3\gamma 2L$ receptors.

CONCLUSION AND IMPLICATIONS

Reducing total mRNA unexpectedly increased δ subunit incorporation into receptors on oocyte plasma membranes. Our results favour homologous locations for δ and $\gamma 2L$ subunits in $\alpha 1\beta 3\gamma 2/\delta$ GABA_A receptors.

Abbreviations

ETO, etomidate; MWC, Monod–Wyman–Changeux; THDOC, tetrahydrodeoxycorticosterone

Introduction

GABA_A receptors are ligand-gated pentameric chloride ion channels formed by five subunits from among 16 homolo-

gous GABA_A receptor subunit subtypes ($\alpha 1$ – $\alpha 6$, $\beta 1$ – $\beta 3$, $\gamma 1$ – $\gamma 3$, δ , ϵ , π and θ) (Olsen and Sieghart, 2008). GABA_A receptors *in vivo* are predominantly composed of $\alpha\beta\delta$ and $\alpha\beta\gamma$ isoforms (McKernan and Whiting, 1996). The $\alpha\beta\gamma$ receptors are mainly

postsynaptic, mediating phasic neuronal inhibition, whereas $\alpha\beta\delta$ receptors are extrasynaptic, mediating tonic inhibition (Mody and Pearce, 2004; Farrant and Nusser, 2005). The subunit stoichiometry of heterologously expressed $\alpha\beta\gamma$ receptors is well established as $2\alpha:2\beta:1\gamma$ (Chang *et al.*, 1996; Tretter *et al.*, 1997), with a counterclockwise subunit arrangement of $\beta-\alpha-\gamma-\beta-\alpha$ viewed from the extracellular space (Baumann *et al.*, 2001). Several studies of recombinant $\alpha\beta\delta$ receptors are consistent with subunit stoichiometry and arrangement similar to those of $\alpha\beta\gamma$ receptors (Barrera *et al.*, 2008; Botzolakis *et al.*, 2008; Shu *et al.*, 2012). However, variable subunit stoichiometry and arrangements in expressed $\alpha\beta\delta$ receptors have been suggested by quantitative biochemical studies varying the ratio of subunit-encoding DNAs (Wagoner and Czajkowski, 2010) and functional comparison of $\alpha\beta\delta$ receptors formed from free subunits with various concatenated subunit assemblies (Kaur *et al.*, 2009).

GABA_A receptors are positively modulated by many general anaesthetics including propofol, etomidate (ETO), pentobarbital and alphaxalone (Hevers and Luddens, 1998; Feng, 2010; Akk and Steinbach, 2011; Forman and Miller, 2011) and by endogenous neurosteroids such as tetrahydrodeoxycorticosterone (THDOC) (Wohlfarth *et al.*, 2002; Stell *et al.*, 2003; Hosie *et al.*, 2006). Many studies have reported that THDOC increases maximal GABA-activated $\alpha\beta\delta$ receptor-mediated currents (Wallner *et al.*, 2003; Zheleznova *et al.*, 2008; Kaur *et al.*, 2009; Meera *et al.*, 2009; Baker *et al.*, 2010; Baur *et al.*, 2010), with the degree of positive modulation varying from several fold to more than 20-fold. This wide range of effects could reflect variable δ subunit stoichiometry and subunit arrangements producing different numbers and types of THDOC sites (Shu *et al.*, 2012). However, inferences regarding $\alpha\beta\delta$ receptor stoichiometry and arrangement cannot be drawn from THDOC modulation because the structures forming neurosteroid sites on GABA_A receptors remain undefined (Akk *et al.*, 2004; Hosie *et al.*, 2006; Bracamontes *et al.*, 2011). In contrast, R-(+)-ETO is known to modulate $\alpha1\beta2/3\gamma2$ GABA_A receptors selectively via two binding sites located at transmembrane subunit interfaces between α -M1 and β -M3 domains (Li *et al.*, 2006; Chiara *et al.*, 2012). A quantitative model describing ETO modulation of GABA_A receptor activity has been validated for $\alpha1\beta2\gamma2$ L receptors formed from free or concatenated subunits and activated with either full or partial agonists (Rusch *et al.*, 2004; Guitchoyts *et al.*, 2012). ETO also enhances $\alpha4\beta3\delta$ receptor currents (Brown *et al.*, 2002; Meera *et al.*, 2009), although the number and location of its sites in $\alpha\beta\delta$ receptors remain unexplored.

We hypothesized that if the subunit stoichiometries or arrangements of $\alpha1\beta3\gamma2$ and $\alpha1\beta3\delta$ differ, then the number of $\alpha1$ -M1/ $\beta3$ -M3 interfacial ETO sites on both receptor isoforms would differ, resulting in divergent allosteric effects with bound ETO. We heterologously expressed, in *Xenopus* oocytes, $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2$ L receptors formed from free subunits as well as concatenated $\beta3-\alpha1-\delta/\beta3-\alpha1$ receptors (designed to form pentamers with δ in the same position as $\gamma2$ L in $\alpha1\beta3\gamma2$ L receptors) and compared ETO modulation of these receptors using two microelectrode electrophysiology and quantitative allosteric model analysis.

Our results show that in oocyte-expressed $\alpha1\beta3\delta$ receptors formed from free subunits, modulation of maximal GABA-

elicited responses by both ETO and THDOC depends on subunit mRNA ratio and the total mRNA injected. Under expression conditions that optimize allosteric gating modulation, ETO produces similar upward (increased maximum) and leftward (reduced EC₅₀) shifts of GABA concentration-response curves for both free subunit $\alpha1\beta3\delta$ and concatenated $\beta3-\alpha1-\delta/\beta3-\alpha1$ receptors. Quantitative allosteric shifts calculated for ETO modulation of both $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2$ L receptors did not differ significantly. Our results indicate that conditions leading to efficient δ subunit incorporation into *Xenopus* oocyte-expressed GABA_A receptors differ from those for $\gamma2$ L incorporation. They also favour the hypothesis that $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2$ L receptors have a similar number and configuration of ETO sites formed by $\beta3$ and $\alpha1$ subunits.

Methods

Animals and oocyte harvest

Animal procedures were approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital. Frogs were purchased from Xenopus 1, Dexter, Michigan, and ~20 frogs were used in these experiments. They were kept in fresh water at 18°C (1–2 frogs per cage). The room was on a 12-h/12-h light/dark cycle (light on 07 h–19 h) in a facility that was supervised by veterinarians.

Oocytes were harvested through a mini-laparotomy with frogs anaesthetized, by immersion in water (~20°C) containing 0.1% tricaine (Sigma-Aldrich, St. Louis, MO, USA). After 15–20 min, the depth of anaesthesia was assessed by pinching the abdomen and lower limbs of the frog with forceps to see if any movement, such as kicking, occurs. The laparotomy was performed only after adequate anaesthesia was attained, based on lack of leg pinch responses. Oocytes were treated with type II collagenase (3.3 mg·mL⁻¹) (Worthington Biochemical, Lakewood, NJ, USA) for 3 h, washed and maintained in ND96 solution (see below for ionic concentrations) supplemented with antibiotics: gentamicin (0.05 mg·mL⁻¹; Invitrogen, Grand Island, NY, USA), amikacin (100 µg·mL⁻¹; Sigma-Aldrich) and ciprofloxacin (2 mg·mL⁻¹; Sigma-Aldrich). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Expression of recombinant GABA_A receptors in oocytes

The cDNAs encoding human $\alpha1$, rat $\beta3$, δ , $\beta3-\alpha1-\delta$ trimer and $\beta3-\alpha1$ dimer GABA_A receptor subunits were generously provided by Dr Erwin Sigel (Department of Biochemistry and Molecular Medicine, University of Bern, Switzerland). The linker between $\beta3$ and $\alpha1$ subunits is composed of 23 amino acids (Q₄A₃PTGQA₃PA₂Q₅), and that between $\alpha1$ and δ subunits is 10 amino acids (Q₄TGQ₄) (Kaur *et al.*, 2009). These subunit cDNAs as well as cDNAs encoding human $\alpha1$, $\beta3$ and $\gamma2$ L subunits were subcloned into pCMV or pcDNA3.1 vectors. Receptor nomenclature follows BJP's *Concise Guide to PHARMACOLOGY* (Alexander *et al.*, 2013). mRNA was synthesized using mMESSAGE mMACHINE kits (Ambion, Austin, TX, USA) from linearized cDNA templates, and a

poly-A tail was added to mRNA [Poly(A) Tailing Kits; Ambion]. Subunit mRNA concentrations were determined spectrophotometrically. mRNAs mixed at different molar ratios were diluted to desired final concentration (0.01–0.1 ng·nL⁻¹) and microinjected into oocytes (50 nL total). Oocytes were incubated at 18°C in ND96 supplemented with antibiotics until used for electrophysiology (24–72 h).

Two electrode voltage clamp electrophysiology

Oocytes were placed in a low-volume (30 μ L) flow chamber continuously perfused by ND96 solution at a rate of 3 mL·min⁻¹. Whole-cell currents were recorded from oocytes using the two electrode voltage clamp technique at room temperature (21–23°C). Recording electrodes were pulled from borosilicate capillary glass (i.d. = 0.68 mm, o.d. = 1.2 mm) (A-M Systems, Sequim, WA, USA). Electrodes were filled with 3 M KCl, and resistance was 1.0–1.4 M Ω .

Oocytes were voltage clamped at –50 or –70 mV (model OC-725C; Warner Instruments, Hamden, CT, USA). Scaled current output was low-pass filtered at 1 kHz, digitized at 100 Hz via a Digidata 1322A interface (Molecular Devices, Sunnyvale, CA, USA) and recorded using Clampex 9.2 software (Molecular Devices). GABA and/or modulator solutions were delivered for 25 s using a custom-built computer-actuated valve controller. A washout interval between consecutive applications ranged from 1 to 5 min depending on the drug concentrations applied. To study the effect of a modulator (ETO, THDOC or zinc) on free or concatenated $\alpha 1\beta 3\delta$ receptors with different molar ratios and total mRNA amounts, the modulator was pre-applied for 30 s before co-application of GABA (1 mM) and modulator. GABA concentration-response curves were examined in the absence or presence of 3.2 μ M ETO for $\alpha 1\beta 3\delta$ receptors expressed from diluted free or concatenated subunits. ETO was not pre-applied when GABA concentration-response curves were performed. Spontaneous channel activity was investigated by applying a GABA_A receptor antagonist picrotoxin (2 mM) in the absence of GABA.

Chemicals and solutions

R-(+)-ETO [2 mg·mL⁻¹ in 35% propylene glycol/water (v v⁻¹) formulation] was obtained from Hospira Inc. (Lake Forest, IL, USA), and other chemicals were purchased from either Sigma-Aldrich or Fisher Scientific (Fair Lawn, NJ, USA), unless otherwise mentioned. ND96 solution was composed of (in mM) 100 NaCl, 2 KCl, 1 CaCl₂, 0.8 MgCl₂, 1 EGTA and 10 HEPES, pH 7.5. EGTA was omitted from ND96 when ZnCl₂ was used in the experiments. GABA (1 M) and ZnCl₂ (10 mM) stock solutions were prepared in water, and THDOC stock (10 mM) was prepared in DMSO. Solutions were prepared by diluting the stock solution with ND96 on the day of the experiment. The final concentration of DMSO in experimental solutions was 0.01%. ETO was diluted into ND96. Picrotoxin (2 mM) was dissolved in ND96 by prolonged gentle shaking.

Data analysis

Currents were analysed offline using Clampfit 9.2 (Molecular Devices). Enhancement of saturating GABA (1 mM)-evoked currents by ETO (3.2 μ M) or THDOC (1 μ M) was determined using the ratio of the peak current elicited by co-application

of GABA and ETO (THDOC) and that evoked by GABA alone. For GABA concentration–response data, all currents were normalized to control currents evoked by 1 mM GABA. Normalized concentration–response data were fitted using non-linear least squares with a variable slope logistic equation: $I = I_{\max} / (1 + 10^{(\text{LogEC}_{50} - \text{Log[GABA]}) \times \text{Hill slope}})$. I is the normalized peak current. $I_{\max}$ is the maximal peak current. EC₅₀ is the GABA concentration eliciting 50% of maximal response. Data are reported as mean $\pm$ SEM, unless otherwise noted. THDOC, ETO or zinc modulation results in various receptors expressed under different conditions were compared using one-way ANOVA with a *post hoc* Tukey's multiple comparison test. Statistical significance was inferred if P was less than 0.05.

Allosteric modelling

Allosteric gating shifts caused by ETO were quantified using a modification of the approach we have previously described for fitting a Monod–Wyman–Changeux (MWC) allosteric co-agonist model (Stewart *et al.*, 2013) to estimated open probability values. Fits were performed using Origin 6.1 (Microcal, Northampton, MA, USA).

Estimated open probability ($P_{\text{open}}^{\text{est}}$) was calculated by explicitly adding average spontaneous activity ($\frac{I_{\text{PTX}}}{I_{\max}^{\text{GABA}}}$) to normalized activated currents ($\frac{I}{I_{\max}^{\text{GABA}}}$) and renormalizing to the full range of open probability, bracketed by maximal ETO-enhanced current ($\frac{I_{\max}^{\text{GABA+ETO}}}{I_{\max}^{\text{GABA}}}$; $P_{\text{open}} = 1.0$) and picrotoxin-blocked basal current ($P_{\text{open}} = 0$):

$$P_{\text{open}}^{\text{est}} = \frac{\frac{I}{I_{\max}^{\text{GABA}}} + \frac{I_{\text{PTX}}}{I_{\max}^{\text{GABA}}}}{\frac{I_{\max}^{\text{GABA+ETO}}}{I_{\max}^{\text{GABA}}} + \frac{I_{\text{PTX}}}{I_{\max}^{\text{GABA}}}} \quad (1)$$

For $\alpha 1\beta 3\delta$ (1:1:3, 0.5 ng) and $\beta 3\text{-}\alpha 1\text{-}\delta/\beta 3\text{-}\alpha 1$ (1:1, 1 ng) receptors in this study, picrotoxin did not shift basal currents ($n = 4$ for each receptor), indicating a lack of detectable spontaneous channel activity. Thus, $\frac{I_{\text{PTX}}}{I_{\max}^{\text{GABA}}}$ was set at 0. In concentration–response studies of $\alpha 1\beta 3\delta$ receptors, maximal GABA alone produced responses that were 14- to 20-fold lower than those with GABA plus 3.2 μ M ETO. We also estimated maximal P_{open} in the presence of 3.2 μ M ETO by comparing responses to those with GABA + 10 μ M ETO, which were assumed to represent response of all activatable receptors ($P_{\text{open}} = 1.0$) because 30 μ M ETO did not further enhance currents. For $\alpha 1\beta 3\delta$ (1:1:3, 0.5 ng) receptors, 3.2 μ M ETO enhanced GABA responses significantly less than 10 μ M ($n = 6$; $P < 0.05$), and the ratio of $\frac{I_{\max}^{\text{GABA+3.2ETO}}}{I_{\max}^{\text{GABA+10ETO}}}$ was 0.6 ± 0.11 (mean $\pm$ SD). For $\beta 3\text{-}\alpha 1\text{-}\delta/\beta 3\text{-}\alpha 1$ (1:1, 1 ng) receptors, this ratio was 0.76 ± 0.097 ($n = 5$; $P < 0.05$). Multiplying these results with ratios for $\frac{I_{\max}^{\text{GABA}}}{I_{\max}^{\text{GABA+3.2ETO}}}$ produced GABA efficacy estimates ($\frac{I_{\max}^{\text{GABA}}}{I_{\max}^{\text{GABA+10ETO}}}$) for both free and concatenated $\alpha 1\beta 3\delta$ receptors ranging from 0.03 to 0.05.

Non-linear least squares fits to the MWC two-state co-agonist mechanism (Equation 2a) used average $P_{\text{open}}^{\text{est}}$ data from GABA concentration responses with and without ETO. Both [GABA] and [ETO] are independent continuous variables:

$$P_{\text{open}} = \frac{1}{1 + L_0 \times \left(\frac{1 + [\text{GABA}]/K_G}{1 + [\text{GABA}]/cK_G} \right)^2 \left(\frac{1 + [\text{ETO}]/K_E}{1 + [\text{ETO}]/dK_E} \right)^{nE}} \quad (2a)$$

L_0 in Equation 2a is a dimensionless basal equilibrium gating parameter, approximately P_0^{-1} . In this study, L_0 was set at 25 000 based on previous estimates for $\alpha 1\beta 2\gamma 2\text{L}$ GABA_A receptors (Rusch *et al.*, 2004; Stewart *et al.*, 2013). K_G and K_E are dissociation constants for GABA and ETO interactions with closed receptors, whereas c and d are single-site efficacy parameters for GABA and ETO respectively. The parameter nE represents the number of ETO sites, usually 2.

We modified the fitting procedure, transforming Equation 2a into a bimodal function with global parameters for GABA affinity (K_G) and efficacy (c), and a single allosteric shift parameter, D , for the ETO concentration we tested:

$$P_{\text{open}} = \frac{1}{1 + L_0 \times \left(\frac{1 + [\text{GABA}]/K_G}{1 + [\text{GABA}]/cK_G} \right)^2 \left(\frac{1 + [\text{ETO}]/10^{-6}}{1 + [\text{ETO}]/10^{-6}D} \right)} \quad (2b)$$

In the modified fitting procedure, [ETO] was treated as a binary variable with a value of 0 when absent and 1 when present. The value of $K_E = 10^{-6}$ was chosen so that in the presence of ETO, $[\text{ETO}]/K_E = 10^6$, mimicking full ETO site occupancy. We also set $nE = 1$. With these conditions, when ETO is absent, $[\text{ETO}] = 0$, $\left(\frac{1 + [\text{ETO}]/10^{-6}}{1 + [\text{ETO}]/10^{-6}D} \right) = 1$ and Equation 2b describes the concentration response of GABA alone acting at its two agonist sites:

$$P_{\text{open}} = \frac{1}{1 + L_0 \times \left(\frac{1 + [\text{GABA}]/K_G}{1 + [\text{GABA}]/cK_G} \right)^2} \quad (3a)$$

When ETO is present, $[\text{ETO}] = 1$, $[\text{ETO}]/K_E = 10^6$, $\left(\frac{1 + [\text{ETO}]/10^{-6}}{1 + [\text{ETO}]/10^{-6}D} \right) \approx D$ and Equation 2b becomes:

$$P_{\text{open}} = \frac{1}{1 + L_0 \times D \times \left(\frac{1 + [\text{GABA}]/K_G}{1 + [\text{GABA}]/cK_G} \right)^2} \quad (3b)$$

Thus, when $P_{\text{open}}^{\text{est}}$ data for GABA concentration-responses in both the absence and presence of ETO are simultaneously fitted using the modified procedure, we obtain estimates (mean $\pm$ SEM) for K_G , c and D , the latter representing the allosteric shift produced by the experimental ETO concentration. This calculation makes no assumptions about, nor derives estimates for, the affinity, efficacy or number of ETO sites. Its purpose is to quantify allosteric shift by a given modulator concentration, enabling comparisons between receptors where GABA efficacy differs widely, as it does for $\alpha 1\beta 3\gamma 2$ versus $\alpha 1\beta 3\delta$ receptors.

Results

Allosteric modulation of $\alpha 1\beta 3\delta$ GABA_A receptors formed from free or concatenated subunits with different molar ratios and total mRNA amounts

We first examined the modulation by THDOC of $\alpha 1\beta 3$ and $\alpha 1\beta 3\delta$ GABA_A receptors formed from free or concatenated

subunits with different molar ratios and total mRNA amounts, including $\alpha 1\beta 3(1:1, 5 \text{ ng})$, $\alpha 1\beta 3\delta(1:1:1, 5 \text{ ng})$, $\alpha 1\beta 3\delta(1:1:1, 0.5 \text{ ng})$, $\alpha 1\beta 3\delta(1:1:3, 5 \text{ ng})$, $\alpha 1\beta 3\delta(1:1:3, 0.5 \text{ ng})$, $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1(1:1, 5 \text{ ng})$ and $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1(1:1, 1 \text{ ng})$. THDOC at $1 \mu\text{M}$ potentiated saturating GABA (1 mM)-evoked currents for all the receptors tested (Figure 1A and B). ANOVA analysis (Figure 1B) indicated that THDOC enhancement was

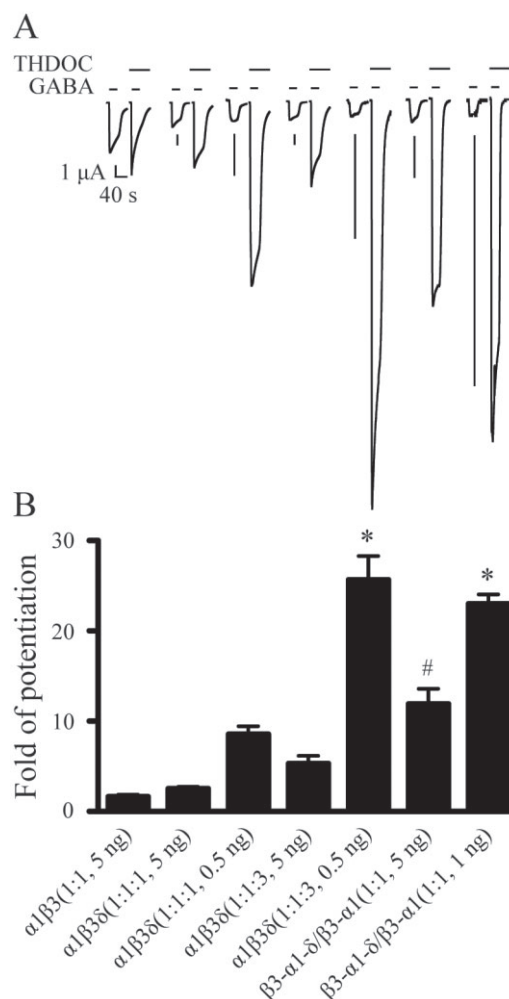


Figure 1

Modulation by THDOC of $\alpha 1\beta 3\delta$ GABA_A receptors expressed from free or concatenated subunits with different molar ratios and total mRNA amounts. (A) Representative current traces evoked by saturating concentration of GABA (1 mM) as well as co-application of 1 mM GABA and $1 \mu\text{M}$ THDOC with THDOC pre-applied for 30 s. Receptors were expressed by injection of GABA_A receptor subunits into oocytes with different molar ratios and total mRNA amounts (see corresponding receptors in panel B). The horizontal bars above each current trace indicate the application of GABA and THDOC respectively. The timescale is the same, and the amplitude scale is $1 \mu\text{A}$ for all current traces. (B) The fold of potentiation by THDOC for $\alpha 1\beta 3$ receptors as well as $\alpha 1\beta 3\delta$ receptors formed with different molar ratios and total mRNA amounts injected into oocytes. The error bars represent SEMs. *Significantly different from $\alpha 1\beta 3\delta(1:1:1, 0.5 \text{ ng})$ receptors at $P < 0.001$. #Significantly different from $\alpha 1\beta 3(1:1, 5 \text{ ng})$, $\alpha 1\beta 3\delta(1:1:1, 5 \text{ ng})$ and $\alpha 1\beta 3\delta(1:1:3, 5 \text{ ng})$ receptors at $P < 0.01$ or 0.001 .

not significantly different for receptors expressed with three mRNA mixes at 5 ng per oocyte: $\alpha 1\beta 3(1:1, 5 \text{ ng})$, 1.6 ± 0.2 , ($n = 6$); $\alpha 1\beta 3\delta(1:1:1, 5 \text{ ng})$, 2.5 ± 0.2 , ($n = 6$); and $\alpha 1\beta 3\delta(1:1:3, 5 \text{ ng})$, 5.3 ± 0.8 , ($n = 9$). Significantly more THDOC potentiation was observed for $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1(1:1, 5 \text{ ng})$ receptors (11.9 ± 1.7 , $n = 9$; $P < 0.01$). Oocytes injected with 0.5 ng $\alpha 1\beta 3\delta(1:1:1, 0.5 \text{ ng})$ mRNAs displayed greater THDOC potentiation (8.6 ± 0.9 , $n = 6$) than that in $\alpha 1\beta 3(1:1, 5 \text{ ng})$ receptors ($P < 0.05$), but not statistically different from $\alpha 1\beta 3\delta(1:1:1, 5 \text{ ng})$, $\alpha 1\beta 3\delta(1:1:3, 5 \text{ ng})$ and $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1(1:1, 5 \text{ ng})$ receptors. The largest THDOC potentiations were observed in $\alpha 1\beta 3\delta(1:1:3, 0.5 \text{ ng})$ (25.7 ± 2.6 , $n = 7$) and $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1(1:1, 1 \text{ ng})$ receptors (23.0 ± 1.0 , $n = 6$), both significantly greater ($P < 0.001$) than that in $\alpha 1\beta 3\delta(1:1:1, 0.5 \text{ ng})$ receptors (Figure 1B).

We next examined potentiation by 3.2 μM ETO in oocytes injected with the same mRNA mixtures tested against THDOC. Allosteric modulation by ETO in this second set of oocytes was similar to that by THDOC (Figure 2A and B). ETO produced significantly greater potentiation in $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1(1:1, 5 \text{ ng})$ receptors (14.6 ± 2.0 , $n = 12$) than in $\alpha 1\beta 3(1:1, 5 \text{ ng})$ (1.7 ± 0.2 , $n = 8$; $P < 0.01$), $\alpha 1\beta 3\delta(1:1:1, 5 \text{ ng})$ (3.2 ± 0.3 , $n = 9$; $P < 0.01$) and $\alpha 1\beta 3\delta(1:1:3, 5 \text{ ng})$ receptors (4.4 ± 0.2 , $n = 9$; $P < 0.05$). Reducing total mRNA injected for $\alpha 1\beta 3\delta$ receptors (0.5–1.0 ng) also produced more ETO potentiation. ETO potentiation was greatest in $\alpha 1\beta 3\delta(1:1:3, 0.5 \text{ ng})$ (26.4 ± 4.4 , $n = 9$) and $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1(1:1, 1 \text{ ng})$ receptors (34.4 ± 3.8 , $n = 6$), and both results were significantly higher than those in $\alpha 1\beta 3\delta(1:1:3, 5 \text{ ng})$, $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1(1:1, 5 \text{ ng})$, $\alpha 1\beta 3\delta(1:1:1, 0.5 \text{ ng})$ and $\alpha 1\beta 3\delta(1:1:1, 5 \text{ ng})$ receptors ($P < 0.01$ or 0.001 for all pairs; Figure 2B).

In addition to the positive modulators, we also examined the effect of zinc, a negative GABA_A receptor modulator, on the currents of $\alpha 1\beta 3(1:1, 5 \text{ ng})$, $\alpha 1\beta 3\delta(1:1:3, 0.5 \text{ ng})$ and $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1(1:1, 1 \text{ ng})$ receptors evoked by corresponding EC₅₀ GABA. In line with previous studies in oocytes (Karim *et al.*, 2012; Shu *et al.*, 2012), Zn²⁺ at 1 μM inhibited GABA (3 μM)-induced currents of $\alpha 1\beta 3(1:1, 5 \text{ ng})$ receptors by $87.2 \pm 1.7\%$ ($n = 6$). Zn²⁺ (1 μM) produced significantly less inhibition in both $\alpha 1\beta 3\delta(1:1:3, 0.5 \text{ ng})$ ($32.5 \pm 4.9\%$, $n = 8$; $P < 0.001$) and $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1(1:1, 1 \text{ ng})$ receptors ($27.2 \pm 4.2\%$, $n = 7$; $P < 0.001$).

ETO produces similar upward and leftward shifts of GABA concentration responses for $\alpha 1\beta 3\delta(1:1:3, 0.5 \text{ ng})$ and $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1(1:1, 1 \text{ ng})$ receptors

We examined GABA concentration responses with and without ETO (3.2 μM) in $\alpha 1\beta 3\delta(1:1:3, 0.5 \text{ ng})$, $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1(1:1, 1 \text{ ng})$ and $\alpha 1\beta 3\gamma 2\text{L}(1:1:5, 5 \text{ ng})$ receptors in order to compare ETO modulation of these channels quantitatively. The GABA EC₅₀ for $\alpha 1\beta 3\delta(1:1:3, 0.5 \text{ ng})$ receptors was 10.3 μM ($n = 4$). ETO (3.2 μM) produced an approximately threefold leftward shift (GABA EC₅₀ = 3.7 μM , $n = 4$), and an ~17-fold (17.3 ± 2.0) increase in maximal GABA responses (Figure 3). Concatenated $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1(1:1, 1 \text{ ng})$ receptors displayed a higher GABA EC₅₀ (74.7 μM , $n = 7$), consistent with previous reports (Kaur *et al.*, 2009; Baur *et al.*, 2010). In $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1(1:1, 1 \text{ ng})$ receptors, ETO produced an approximately threefold leftward shift (GABA EC₅₀ = 22.2 μM) and a

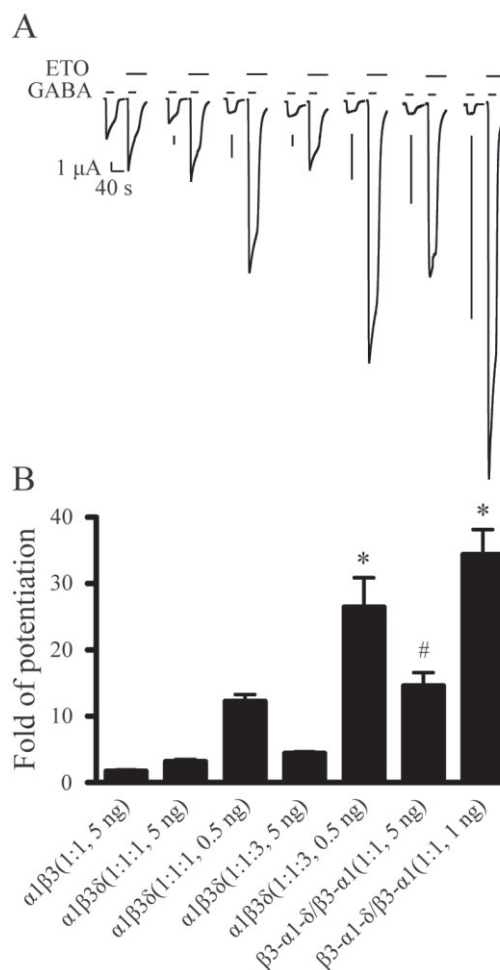


Figure 2

Modulation by ETO of $\alpha 1\beta 3\delta$ GABA_A receptors expressed from free or concatenated subunits with different molar ratios and total mRNA amounts. (A) Representative current traces evoked by saturating concentration of GABA (1 mM) as well as co-application of 1 mM GABA and 3.2 μM ETO with ETO pre-applied for 30 s. Receptors were expressed by injection of GABA_A receptor subunits into oocytes with different molar ratios and total mRNA amounts (see corresponding receptors in panel B). The horizontal bars above each current trace indicate the application of GABA and ETO respectively. The timescale is the same, and the amplitude scale is 1 μA for all current traces. (B) The fold of potentiation by ETO for $\alpha 1\beta 3$ receptors as well as $\alpha 1\beta 3\delta$ receptors formed with different molar ratios and total mRNA amounts injected into oocytes. The error bars represent SEMs. *Significantly different from $\alpha 1\beta 3\delta(1:1:1, 0.5 \text{ ng})$ receptors at $P < 0.01$ or 0.001. #Significantly different from $\alpha 1\beta 3(1:1, 5 \text{ ng})$, $\alpha 1\beta 3\delta(1:1:1, 5 \text{ ng})$ and $\alpha 1\beta 3\delta(1:1:3, 5 \text{ ng})$ receptors at $P < 0.05$ or 0.01.

large (14.0 ± 2.2 -fold) increase in maximal response at high GABA (Figure 4).

ETO effects on GABA concentration–response parameters for $\alpha 1\beta 3\gamma 2\text{L}$ receptors differed from those observed in $\alpha 1\beta 3\delta$ receptors. The GABA EC₅₀ in $\alpha 1\beta 3\gamma 2\text{L}$ receptors was 7.8 μM ($n = 6$), and 3.2 μM ETO produced about a 10-fold leftward shift (GABA EC₅₀ = 0.79 μM , $n = 7$) (Figure 5A). Maximal peak

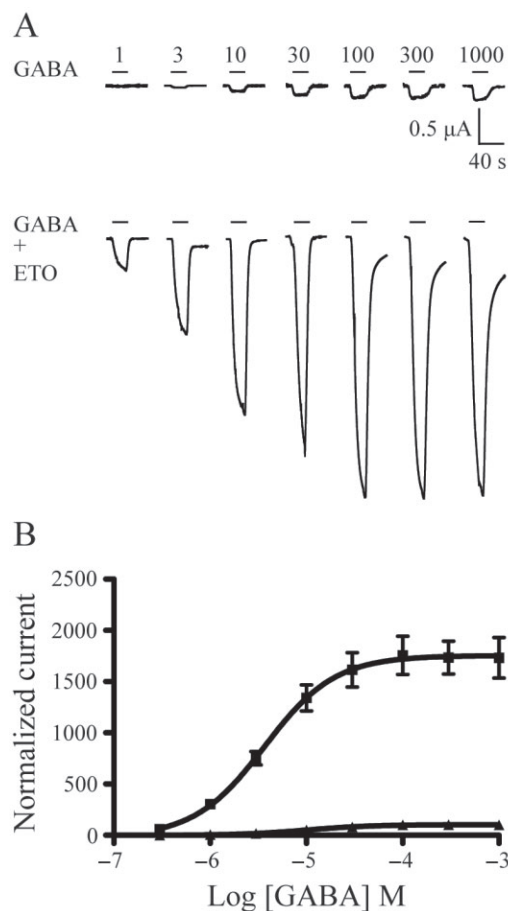


Figure 3

ETO modulation of GABA concentration-response data for $\alpha 1\beta 3\delta(1:1:3, 0.5 \text{ ng})$ receptors. (A) Examples of current traces evoked by increasing concentrations of GABA (in μM) as well as co-application of each concentration of GABA and ETO ($3.2 \mu\text{M}$) for $\alpha 1\beta 3\delta(1:1:3, 0.5 \text{ ng})$ receptors. (B) The concentration-response curves for GABA alone (triangles) and co-application of GABA with $3.2 \mu\text{M}$ ETO (squares) were plotted for $\alpha 1\beta 3\delta(1:1:3, 0.5 \text{ ng})$ receptors. ETO produced upward and leftward shifts of the concentration-response curve for the receptors. The horizontal bar above each current trace indicates GABA application or co-application of GABA and ETO. $n = 4$ cells for GABA or GABA + ETO concentration-response curve. The error bars represent SEMs.

$\alpha 1\beta 3\gamma 2\text{L}$ receptor currents elicited with 1–3 mM GABA plus ETO were 24% greater than those with GABA alone.

ETO produces similar allosteric modulation in $\alpha 1\beta 3\delta$ and $\alpha 1\beta 3\gamma 2\text{L}$ receptors

We quantified ETO-induced allosteric shifts in GABA concentration responses using a modified global fit procedure and an established model that accounts for allosteric modulation of $\alpha 1\beta 2\gamma 2\text{L}$ responses to both full and partial agonists (Rusch *et al.*, 2004). In the modified fit procedure, we treated ETO as a binary variable (0 or 1) and collapsed parameters for ETO affinity (K_E), efficacy (d) and number of sites (nE) into a single allosteric shift parameter, D (see Equations 2b, 3a and 3b in Methods section).

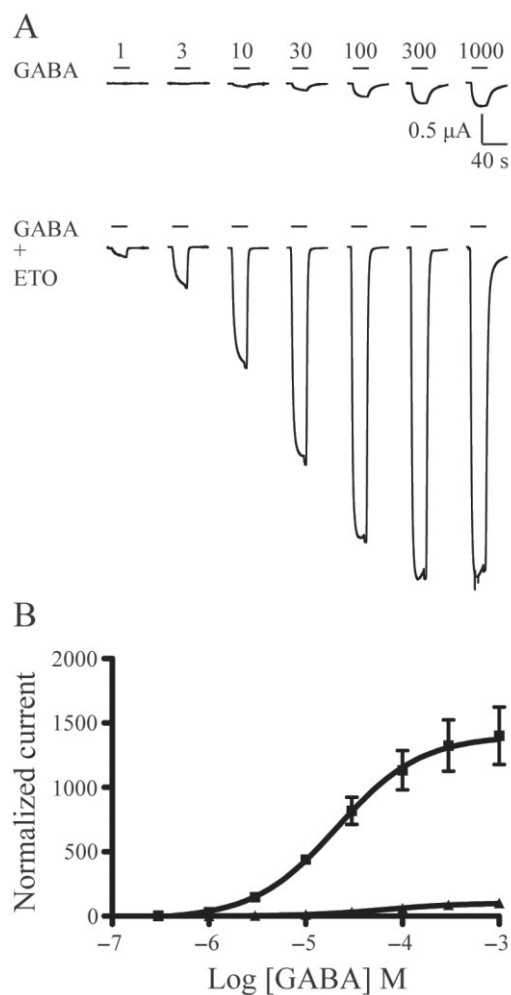


Figure 4

ETO modulation of GABA concentration-response data for $\beta 3\text{-}\alpha 1\text{-}\delta/\beta 3\text{-}\alpha 1(1:1, 1 \text{ ng})$ receptors. (A) Examples of current traces evoked by increasing concentrations of GABA (in μM) as well as co-application of each concentration of GABA and ETO ($3.2 \mu\text{M}$) for $\beta 3\text{-}\alpha 1\text{-}\delta/\beta 3\text{-}\alpha 1(1:1, 1 \text{ ng})$ receptors. (B) The concentration-response curves for GABA alone (triangles) and co-application of GABA with $3.2 \mu\text{M}$ ETO (squares) were plotted for $\beta 3\text{-}\alpha 1\text{-}\delta/\beta 3\text{-}\alpha 1(1:1, 1 \text{ ng})$ receptors. ETO produced upward and leftward shifts of the concentration-response curve for the receptors. The horizontal bar above each current trace indicates GABA application or co-application of GABA and ETO. $n = 7$ cells for GABA or GABA + ETO concentration-response curve. The error bars represent SEMs.

For $\alpha 1\beta 3\gamma 2\text{L}$ receptors, maximal GABA efficacy was estimated at 0.80. Allosteric shift analysis of $\alpha 1\beta 3\gamma 2\text{L}$ $p_{\text{open}}^{\text{pest}}$ values resulted in a good fit ($R^2 = 0.998$) and an allosteric shift factor (D in Equation 3b) of 0.021 (Figure 5B; Table 1).

For comparison of allosteric shifts in $\alpha 1\beta 3\delta$ receptors, $p_{\text{open}}^{\text{pest}}$ was calculated for maximal GABA efficacy values ranging from 0.03 to 0.05. This range is based on both ETO enhancement of responses to maximal GABA alone (Figure 2) and on measurements estimating the efficacy of maximal GABA plus $3.2 \mu\text{M}$ ETO (by comparison with GABA plus $10 \mu\text{M}$ ETO). Allosteric shift parameters for $\alpha 1\beta 3\delta(1:1:3,$

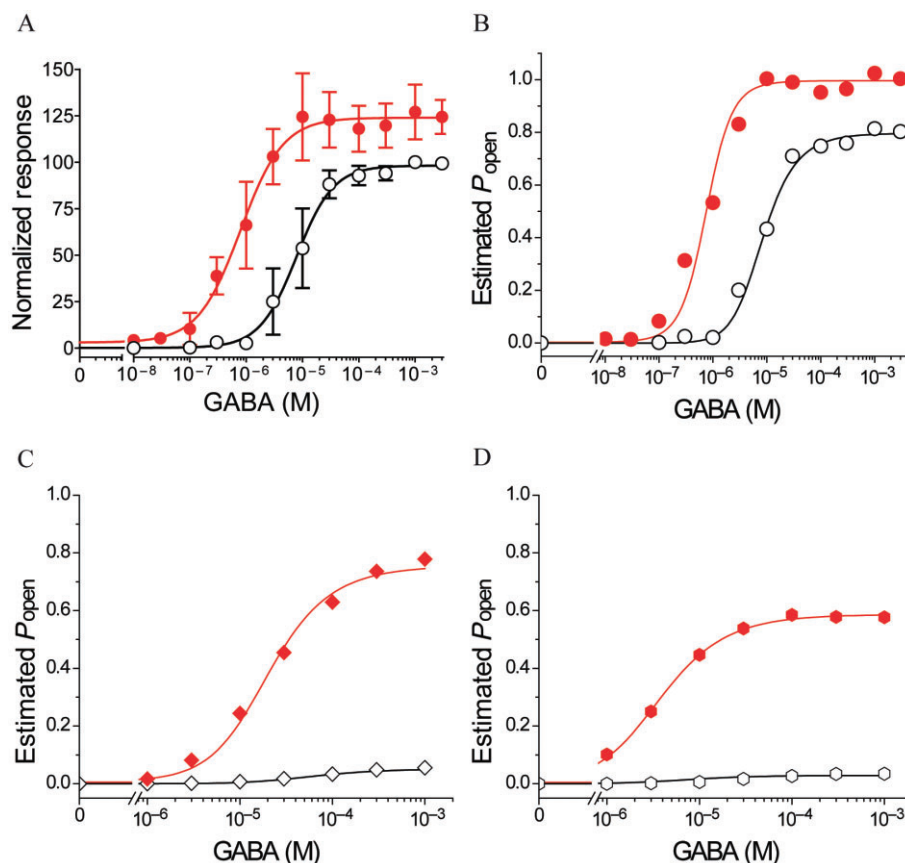


Figure 5

ETO allosteric shift quantified in $\alpha 1\beta 3\gamma 2L$ and $\alpha 1\beta 3\delta$ receptors using MWC co-agonist fits with [ETO] as a binary parameter. (A) Normalized GABA-dependent responses (mean $\pm$ SD; $n \geq 5$) for $\alpha 1\beta 3\gamma 2L$ receptors are plotted. Lines underlying data points are fits to logistic functions. Open symbols are control GABA responses: max = 100 ± 2.5 , $EC_{50} = 7.8$ (5.0–10.3) μM , $nH = 1.3 \pm 0.18$. Solid red symbols are GABA responses in the presence of 3.2 μM ETO: max = 124 ± 3.0 , $EC_{50} = 0.79$ (0.55–1.14) μM , $nH = 1.2 \pm 0.21$. (B–D) Estimated P_{open} was calculated from average data for $\alpha 1\beta 3\gamma 2L$ receptors from panel A (B), $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1$ receptors from Figure 4 (C) and $\alpha 1\beta 3\delta$ receptors formed from diluted free subunits from Figure 3 (D), as described in Methods section. Simultaneous non-linear least squares fits to Equations 3a and 3b were performed as described in Methods section, using [ETO] as a binary parameter (either 0 or 1). (C) Shows $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1$ receptor results with GABA efficacy = 0.05, and (D) shows $\alpha 1\beta 3\delta$ receptor results with GABA efficacy = 0.03. The allosteric shifts associated with 3.2 μM ETO are reported in Table 1 along with other fitted parameters.

0.5 ng) receptors ranged from 0.0072 to 0.021, and the shift parameters for $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1$ (1:1, 1 ng) receptors ranged from 0.018 to 0.035 (Figure 5C and D; Table 1).

Discussion

Although $\alpha\beta\delta$ GABA_A receptors are recognized as important mediators of neurosteroid modulation and general anaesthetic actions (Belelli *et al.*, 2009), no consensus has emerged on the pentameric arrangement of α , β and δ subunits formed from heterologously expressed free subunits, or that in neurons. Expression of concatenated assemblies of GABA_A receptor subunits was instrumental in defining the assembly of $\alpha\beta\gamma$ receptors (Baumann *et al.*, 2001) and has been previously applied to $\alpha\beta\delta$ (Kaur *et al.*, 2009; Sigel *et al.*, 2009; Baur *et al.*, 2010; Shu *et al.*, 2012). However, several issues contribute to a lack of clarity emerging from these studies. First, as discussed below, oocyte expression of free α , β and δ subunits,

more so than for $\alpha\beta\gamma$, apparently results in different subunit arrangements, depending on the subunit subtypes, the mRNA ratio and, as we demonstrate, the total amount of mRNA injected. Thus, the configuration and functional properties of 'control' receptors formed from free subunits may vary among studies, or even within a single study. Second, in most previous studies, comparison of receptors has been based on both GABA sensitivity and neurosteroid modulation. Subunit concatenation is associated with increased GABA EC_{50} s in both $\alpha\beta\gamma$ (Baumann *et al.*, 2002; Steinbach and Akk, 2011) and $\alpha\beta\delta$ receptors (Kaur *et al.*, 2009; Baur *et al.*, 2010), a result we also observed in the current study, making this functional parameter an unreliable comparator. In addition, comparing neurosteroid modulation remains largely empirical because the number and location of neurosteroid sites remain unknown, nor do we know if subunit concatenation affects steroid modulation. We have addressed these issues in two ways. First, we used a criterion of maximized modulator (THDOC and ETO) effects to determine conditions

Table 1

Fitted MWC parameters^a for ETO modulation of GABA activation of $\alpha 1\beta 3\gamma 2L$ and $\alpha 1\beta 3\delta$ receptors

	$\alpha 1\beta 3\gamma 2L$	$\alpha 1\beta 3\delta$	$\beta 3-\alpha 1-\delta/\beta 3-\alpha 1$		
GABA efficacy	0.80	0.03	0.05	0.03	0.05
K_G (μM)	10.3	3.1	7.7	14.8	25.9
c	0.0033	0.037	0.029	0.036	0.027
D	0.021	0.021	0.0072	0.035	0.018

^aNon-linear least squares fits to Equation 2b were performed as described in Methods section, using [ETO] as a binary parameter (either 0 or 1), with L_0 fixed at a value of 25 000, K_E fixed at a value of 1 μM and $nE = 1$. L_0 is a dimensionless parameter describing the basal equilibrium between open and closed receptors (O : C). K_G is the dissociation constant for GABA binding to closed receptors, whereas c is the efficacy parameter for GABA and D is the allosteric shift produced by 3.2 μM ETO. Maximal GABA efficacy values were based on experimental results (see Methods section) and were used to estimate P_{open} values before fitting.

for optimal $\alpha 1\beta 3\delta$ receptor expression in oocytes. Second, to strengthen inferences about the configuration of subunits in $\alpha 1\beta 3\delta$ receptors, we quantified the effects of R-(+)-ETO, a potent stereoselective allosteric modulator that in $\alpha\beta\gamma$ receptors acts selectively at sites formed between transmembrane α -M1 and β -M3 domains. Importantly, concatenation in $\alpha 1\beta 2\gamma 2L$ receptors does not significantly affect ETO modulation (Guitchounts *et al.*, 2012).

Function of oocyte-expressed $\alpha 1\beta 3\delta$ receptors depends on subunit mRNA molar ratios and total amount injected

Previous studies have demonstrated that many GABA_A receptor modulators, including THDOC, potentiate maximal GABA-elicited currents in $\alpha 1\beta\delta$ receptors far more than those in $\alpha 1\beta$ receptors (Wohlfarth *et al.*, 2002; Feng and Macdonald, 2004; 2010; Zheleznova *et al.*, 2008; Lewis *et al.*, 2010). These observations indicate that GABA is a weak partial agonist of δ subunit-containing receptors. However, others have reported little difference in drug (including ETO) modulation of maximal GABA responses in $\alpha 4\beta$ versus $\alpha 4\beta\delta$ receptors (Meera *et al.*, 2009; Lewis *et al.*, 2010), suggesting variable δ incorporation and/or that α subunits also play an important role in $\alpha\beta\delta$ receptor modulation (Jensen *et al.*, 2013). In our current study, we varied subunit mRNA ratios and total amount injected, and found that the resulting patterns of modulation by both THDOC and ETO were similar (Figures 1 and 2). Enhancement of maximal GABA currents in oocytes injected with 5 ng total mRNA encoding free $\alpha 1$, $\beta 3$ and δ subunits was similar to that in oocytes injected with mRNAs for $\alpha 1$ and $\beta 3$. The most likely interpretation, in agreement with others (Borghese and Harris, 2007; Karim *et al.*, 2012; Shu *et al.*, 2012), is that δ subunits do not efficiently incorporate into oocyte-expressed receptors under these conditions, which we have used successfully to express $\alpha\beta\gamma$ receptors.

At 5 ng total mRNA per oocyte, increasing the ratio of δ mRNA threefold relative to α and β did not significantly affect modulator efficacy in our study. However, concatenated $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1(1:1)$ mRNA resulted in greater THDOC and ETO potentiation than $\alpha 1\beta 3\delta(1:1:1)$ or $\alpha 1\beta 3\delta(1:1:3)$, indicating the expected incorporation of δ as reported by others (Kaur *et al.*, 2009; Shu *et al.*, 2012). We also observed that expression of either $\beta-\alpha$ (dimer) or $\beta-\alpha-\delta$ (trimer) alone results in surface receptors that are activated by GABA, as reported previously (Kaur *et al.*, 2009). It is possible that three dimers or two trimers form pentamers with one extra subunit appended, as demonstrated in nicotinic ACh receptors (Zhou *et al.*, 2003; Minier and Sigel, 2004). However, the functional properties of receptors formed from co-expression of dimers and trimers are reportedly similar to those of fully concatenated pentameric receptors (Kaur *et al.*, 2009; Sigel *et al.*, 2009), suggesting that when both dimers and trimers are present, pentamers are preferentially formed from one of each. Our gating modulation results also indicate that free subunit $\alpha 1\beta 3\delta$ receptors best match those formed from the co-expression of concatenated dimers and trimers together.

Unexpectedly, we found that injecting 10-fold less mRNA (0.5 ng) at 1 α :1 β :3 δ mRNA ratio markedly increased THDOC and ETO modulation (and also reduced zinc inhibition). With lower total mRNA, free subunit mRNA ratios also affected modulation; $\alpha 1\beta 3\delta$ (1:1:3, 0.5 ng) receptors displayed more modulation than a 1:1:1 mix. Thus, subunit mRNA ratio apparently affects the stoichiometry and configuration of oocyte surface receptors, consistent with other studies varying the α : β : δ ratios of mRNA in oocytes (Shu *et al.*, 2012) or cDNAs transfected into HEK cells (Botzolakis *et al.*, 2008; Wagoner and Czajkowski, 2010). Reducing total mRNA also enhanced modulation of oocyte-expressed receptors formed from $\beta 3-\alpha 1-\delta$ and $\beta 3-\alpha 1$ concatemers, although less so than with free subunits. Our novel observation that total mRNA affects surface receptor function (and presumably structure) suggests that competition among subunit mRNAs for limited translation capacity, assembly and/or trafficking elements in oocytes may contribute to variable receptor assembly. Further studies are needed to explore these potential mechanisms.

ETO produces similar allosteric modulation for $\alpha 1\beta 3\delta$ and $\alpha 1\beta 3\gamma 2L$ GABA_A receptors

Comparison of ETO effects on GABA-dependent responses in $\alpha 1\beta 3\delta(1:1:3, 0.5 \text{ ng})$ and $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1(1:1, 1 \text{ ng})$ receptors demonstrated similar reductions in GABA EC₅₀ and increases in maximal responses for both receptors. The effects of ETO on GABA-dependent $\alpha 1\beta 3\gamma 2L$ currents appear very different from those in $\alpha 1\beta 3\delta$ receptors, but similar to previous results in $\alpha 1\beta 2\gamma 2L$ receptors formed with both free (Rusch *et al.*, 2004) and concatenated subunits (Guitchounts *et al.*, 2012).

ETO's allosteric effects were also quantified based on a MWC mechanism that accounts for modulation of $\alpha 1\beta 2\gamma 2$ currents elicited with both full and partial agonists (Rusch *et al.*, 2004; Forman, 2012). Allosteric shift analysis of $\alpha 1\beta 3\gamma 2L$ data indicates that 3.2 μM ETO shifts the closed-open equilibrium 48-fold towards open, or about sevenfold for each ETO site. In comparison, $\beta 3-\alpha 1-\delta/\beta 3-\alpha 1$ data indicate a 29- to 55-fold shift in the closed-open equilibrium. Allosteric shifts in free subunit $\alpha 1\beta 3\delta$ receptors (48- to 140-fold)

overlap with the values for $\beta 3$ - $\alpha 1$ - δ / $\beta 3$ - $\alpha 1$ and $\alpha 1\beta 3\gamma 2L$ receptors. The similarity of ETO allosteric shifts contrasts with the dramatic difference in GABA efficacy for $\alpha\beta\gamma$ and $\alpha\beta\delta$ receptors. The similar allosteric shifts are consistent with the presence of two $\alpha 1$ -M1/ $\beta 3$ -M3 inter-subunit ETO sites on each of these receptors, favouring the hypothesis that the subunit arrangement of $\alpha 1\beta 3\delta$ receptors is $\beta 3$ - $\alpha 1$ - δ - $\beta 3$ - $\alpha 1$, similar to $\alpha 1\beta 3\gamma 2L$.

Our inferences regarding the arrangement of $\alpha 1\beta 3\delta$ subunits are not conclusive. The range of allosteric shift estimates for $\alpha 1\beta 3\delta$ reflects the uncertain gating efficacy of GABA, which appears to be a very weak partial agonist in these receptors (Table 1). Nonetheless, these estimates are consistent with previous reports that neurosteroids increase maximal GABA responses in oocyte-expressed $\alpha 1\beta 2/3\delta$ by 20-fold or more (Zheleznova *et al.*, 2008; Kaur *et al.*, 2009). Given the low efficacy of GABA, we expected the fitted MWC model resting state dissociation constant, K_G , to be close to EC_{50} . However, fitted K_G values (Table 1) are consistently lower than GABA EC_{50} for $\alpha 1\beta 3\delta$ (Figures 3 and 4). This discrepancy decreased as GABA efficacy was increased during analysis. Alternative structural hypotheses may also be consistent with our results. ETO activates $\beta 3$ homo-oligomeric receptors (Cestari *et al.*, 1996) and photolabel derivatives apparently bind at $\beta 3$ -M1/ $\beta 3$ -M3 transmembrane interfaces (Chiara *et al.*, 2012). Thus, configurations of 2 $\alpha 1$, 2 $\beta 3$ and 1 δ that form $\beta 3$ -M1/ $\beta 3$ -M3 interfaces might be modulated similarly to those with two $\alpha 1$ -M1/ $\beta 3$ -M3 sites. It is also conceivable, given that δ is phylogenetically closer to β than to $\gamma 2$, that δ may form ETO sites with adjacent α or β subunits. Combining concatenated subunit assemblies with binding site mutations known to alter ETO sensitivity will be informative in testing these alternative structures.

Conclusions

In *Xenopus* oocytes, functional expression of cell surface $\alpha 1\beta 3\delta$ GABA_A receptors is influenced both by the ratio of subunit mRNAs and by the total amount of mRNA. Surprisingly, reducing total mRNA promotes the incorporation of δ subunits into receptors. Concatenated $\alpha\beta\delta$ subunit assemblies also enhance the incorporation of δ . Analysis using an established allosteric model showed that ETO has quantitatively similar modulatory effects in $\alpha\beta\delta$ and $\alpha\beta\gamma$ receptors. Our results favour the hypothesis that the arrangements of $\alpha 1\beta 3\delta$ and $\alpha 1\beta 3\gamma 2L$ subunits are similar.

Acknowledgements

We thank Mayo Hotta for preliminary studies using reduced mRNA injection and Dr Kunpeng Liu for helpful discussion. This work was supported by P01GM058448 and R01GM089745 to S. A. F.

Conflict of interest

None.

References

- Akk G, Steinbach JH (2011). Structural studies of the actions of anesthetic drugs on the γ -aminobutyric acid type A receptor. *Anesthesiology* 115: 1338–1348.
- Akk G, Bracamontes JR, Covey DE, Evers A, Dao T, Steinbach JH (2004). Neuroactive steroids have multiple actions to potentiate GABA_A receptors. *J Physiol* 558: 59–74.
- Alexander SPH *et al.* (2013). The Concise Guide to PHARMACOLOGY 2013/14: Overview. *Br J Pharmacol* 170: 1449–1867.
- Baker C, Sturt BL, Bamber BA (2010). Multiple roles for the first transmembrane domain of GABA_A receptor subunits in neurosteroid modulation and spontaneous channel activity. *Neurosci Lett* 473: 242–247.
- Barrera NP, Betts J, You H, Henderson RM, Martin IL, Dunn SM *et al.* (2008). Atomic force microscopy reveals the stoichiometry and subunit arrangement of the $\alpha 4\beta 3\delta$ GABA_A receptor. *Mol Pharmacol* 73: 960–967.
- Baumann SW, Baur R, Sigel E (2001). Subunit arrangement of γ -aminobutyric acid type A receptors. *J Biol Chem* 276: 36275–36280.
- Baumann SW, Baur R, Sigel E (2002). Forced subunit assembly in $\alpha 1\beta 2\gamma 2$ GABA_A receptors. Insight into the absolute arrangement. *J Biol Chem* 277: 46020–46025.
- Baur R, Kaur KH, Sigel E (2010). Diversity of structure and function of $\alpha 1\alpha 6\beta 3\delta$ GABA_A receptors: comparison with $\alpha 1\beta 3\delta$ and $\alpha 6\beta 3\delta$ receptors. *J Biol Chem* 285: 17398–17405.
- Belelli D, Harrison NL, Maguire J, Macdonald RL, Walker MC, Cope DW (2009). Extrasynaptic GABA_A receptors: form, pharmacology, and function. *J Neurosci* 29: 12757–12763.
- Borghese CM, Harris RA (2007). Studies of ethanol actions on recombinant δ -containing γ -aminobutyric acid type A receptors yield contradictory results. *Alcohol* 41: 155–162.
- Botzolakis EJ, Stanic AK, Gurba KN, Lagrange AH, Feng HJ, Hu NN *et al.* (2008). Flow cytometric analysis of GABA_A receptor surface expression: evidence that $\alpha\beta\gamma$ and $\alpha\beta\delta$ isoforms have similar subunit stoichiometries and arrangements. *Soc Neurosci Abstr* 34: 427.14.
- Bracamontes J, McCollum M, Esch C, Li P, Ann J, Steinbach JH *et al.* (2011). Occupation of either site for the neurosteroid allopregnanolone potentiates the opening of the GABA_A receptor induced from either transmitter binding site. *Mol Pharmacol* 80: 79–86.
- Brown N, Kerby J, Bonnert TP, Whiting PJ, Wafford KA (2002). Pharmacological characterization of a novel cell line expressing human $\alpha 4\beta 3\delta$ GABA_A receptors. *Br J Pharmacol* 136: 965–974.
- Cestari IN, Uchida I, Li L, Burt D, Yang J (1996). The agonistic action of pentobarbital on GABA_A β -subunit homomeric receptors. *Neuroreport* 7: 943–947.
- Chang Y, Wang R, Barot S, Weiss DS (1996). Stoichiometry of a recombinant GABA_A receptor. *J Neurosci* 16: 5415–5424.
- Chiara DC, Dostalova Z, Jayakar SS, Zhou X, Miller KW, Cohen JB (2012). Mapping general anesthetic binding site(s) in human $\alpha 1\beta 3$ γ -aminobutyric acid type A receptors with [³H]TDBzl-etomidate, a photoreactive etomidate analogue. *Biochemistry* 51: 836–847.
- Farrant M, Nusser Z (2005). Variations on an inhibitory theme: phasic and tonic activation of GABA_A receptors. *Nat Rev Neurosci* 6: 215–229.

- Feng HJ (2010). Allosteric modulation of $\alpha\beta\delta$ GABA_A receptors. *Pharmaceuticals* 3: 3461–3477. Available at: <http://www.mdpi.com/journal/pharmaceuticals> (accessed 11/3/2010).
- Feng HJ, Macdonald RL (2004). Proton modulation of $\alpha1\beta3\delta$ GABA_A receptor channel gating and desensitization. *J Neurophysiol* 92: 1577–1585.
- Feng HJ, Macdonald RL (2010). Barbiturates require the N terminus and first transmembrane domain of the δ subunit for enhancement of $\alpha1\beta3\delta$ GABA_A receptor currents. *J Biol Chem* 285: 23614–23621.
- Forman SA (2012). Monod–Wyman–Changeux allosteric mechanisms of action and the pharmacology of etomidate. *Curr Opin Anaesthesiol* 25: 411–418.
- Forman SA, Miller KW (2011). Anesthetic sites and allosteric mechanisms of action on Cys-loop ligand-gated ion channels. *Can J Anaesth* 58: 191–205.
- Guitchoyants G, Stewart DS, Forman SA (2012). Two etomidate sites in $\alpha1\beta2\gamma2$ γ -aminobutyric acid type A receptors contribute equally and noncooperatively to modulation of channel gating. *Anesthesiology* 116: 1235–1244.
- Hevers W, Luddens H (1998). The diversity of GABA_A receptors. Pharmacological and electrophysiological properties of GABA_A channel subtypes. *Mol Neurobiol* 18: 35–86.
- Hosie AM, Wilkins ME, da Silva HM, Smart TG (2006). Endogenous neurosteroids regulate GABA_A receptors through two discrete transmembrane sites. *Nature* 444: 486–489.
- Jensen ML, Wafford KA, Brown AR, Belelli D, Lambert JJ, Mirza NR (2013). A study of subunit selectivity, mechanism and site of action of the δ selective compound 2 (DS2) at human recombinant and rodent native GABA_A receptors. *Br J Pharmacol* 168: 1118–1132.
- Karim N, Wellendorph P, Absalom N, Bang LH, Jensen ML, Hansen MM *et al.* (2012). Low nanomolar GABA effects at extrasynaptic $\alpha4\beta1/\beta3\delta$ GABA_A receptor subtypes indicate a different binding mode for GABA at these receptors. *Biochem Pharmacol* 84: 549–557.
- Kaur KH, Baur R, Siegel E (2009). Unanticipated structural and functional properties of δ -subunit-containing GABA_A receptors. *J Biol Chem* 284: 7889–7896.
- Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: reporting *in vivo* experiments: the ARRIVE guidelines. *Br J Pharmacol* 160: 1577–1579.
- Lewis RW, Mabry J, Polisar JG, Eagen KP, Ganem B, Hess GP (2010). Dihydropyrimidinone positive modulation of δ -subunit-containing γ -aminobutyric acid type A receptors, including an epilepsy-linked mutant variant. *Biochemistry* 49: 4841–4851.
- Li GD, Chiara DC, Sawyer GW, Husain SS, Olsen RW, Cohen JB (2006). Identification of a GABA_A receptor anesthetic binding site at subunit interfaces by photolabeling with an etomidate analog. *J Neurosci* 26: 11599–11605.
- McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. *Br J Pharmacol* 160: 1573–1576.
- McKernan RM, Whiting PJ (1996). Which GABA_A-receptor subtypes really occur in the brain? *Trends Neurosci* 19: 139–143.
- Meera P, Olsen RW, Otis TS, Wallner M (2009). Etomidate, propofol and neurosteroid THDOC increase the GABA efficacy of recombinant $\alpha4\beta3\delta$ and $\alpha4\beta3$ GABA_A receptors expressed in HEK cells. *Neuropharmacology* 56: 155–160.
- Minier F, Siegel E (2004). Techniques: use of concatenated subunits for the study of ligand-gated ion channels. *Trends Pharmacol Sci* 25: 499–503.
- Mody I, Pearce RA (2004). Diversity of inhibitory neurotransmission through GABA_A receptors. *Trends Neurosci* 27: 569–575.
- Olsen RW, Sieghart W (2008). International Union of Pharmacology. LXX. Subtypes of γ -aminobutyric acid_A receptors: classification on the basis of subunit composition, pharmacology, and function. Update. *Pharmacol Rev* 60: 243–260.
- Rusch D, Zhong H, Forman SA (2004). Gating allostereism at a single class of etomidate sites on $\alpha1\beta2\gamma2$ GABA_A receptors accounts for both direct activation and agonist modulation. *J Biol Chem* 279: 20982–20992.
- Shu HJ, Bracamontes J, Taylor A, Wu K, Eaton MM, Akk G *et al.* (2012). Characteristics of concatemeric GABA_A receptors containing $\alpha4/\delta$ subunits expressed in *Xenopus* oocytes. *Br J Pharmacol* 165: 2228–2243.
- Siegel E, Kaur KH, Luscher BP, Baur R (2009). Use of concatamers to study GABA_A receptor architecture and function: application to δ -subunit-containing receptors and possible pitfalls. *Biochem Soc Trans* 37: 1338–1342.
- Steinbach JH, Akk G (2011). Use of concatamers of ligand-gated ion channel subunits to study mechanisms of steroid potentiation. *Anesthesiology* 115: 1328–1337.
- Stell BM, Brickley SG, Tang CY, Farrant M, Mody I (2003). Neuroactive steroids reduce neuronal excitability by selectively enhancing tonic inhibition mediated by δ subunit-containing GABA_A receptors. *Proc Natl Acad Sci U S A* 100: 14439–14444.
- Stewart DS, Hotta M, Desai R, Forman SA (2013). State-dependent etomidate occupancy of its allosteric agonist sites measured in a cysteine-substituted GABA_A receptor. *Mol Pharmacol* 83: 1200–1208.
- Tretter V, Ehya N, Fuchs K, Sieghart W (1997). Stoichiometry and assembly of a recombinant GABA_A receptor subtype. *J Neurosci* 17: 2728–2737.
- Wagoner KR, Czajkowski C (2010). Stoichiometry of expressed $\alpha4\beta2\delta$ γ -aminobutyric acid type A receptors depends on the ratio of subunit cDNA transfected. *J Biol Chem* 285: 14187–14194.
- Wallner M, Hancher HJ, Olsen RW (2003). Ethanol enhances $\alpha4\beta3\delta$ and $\alpha6\beta3\delta$ γ -aminobutyric acid type A receptors at low concentrations known to affect humans. *Proc Natl Acad Sci U S A* 100: 15218–15223.
- Wohlfarth KM, Bianchi MT, Macdonald RL (2002). Enhanced neurosteroid potentiation of ternary GABA_A receptors containing the δ subunit. *J Neurosci* 22: 1541–1549.
- Zheleznova N, Sedelnikova A, Weiss DS (2008). $\alpha1\beta2\delta$, a silent GABA_A receptor: recruitment by tracazolote and neurosteroids. *Br J Pharmacol* 153: 1062–1071.
- Zhou Y, Nelson ME, Kuryatov A, Choi C, Cooper J, Lindstrom J (2003). Human $\alpha4\beta2$ acetylcholine receptors formed from linked subunits. *J Neurosci* 23: 9004–9015.