

RESEARCH ARTICLE

# The Direct Actions of GABA, 2'-Methoxy-6-Methylflavone and General Anaesthetics at $\beta 3\gamma 2L$ GABA<sub>A</sub> Receptors: Evidence for Receptors with Different Subunit Stoichiometries

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## Abstract

2'-Methoxy-6-methylflavone (2'MeO6MF) is an anxiolytic flavonoid which has been shown to display GABA<sub>A</sub> receptor (GABA<sub>A</sub>R)  $\beta 2/3$ -subunit selectivity, a pharmacological profile similar to that of the general anaesthetic etomidate. Electrophysiological studies suggest that the full agonist action of 2'MeO6MF at  $\alpha 2\beta 3\gamma 2L$  GABA<sub>A</sub>Rs may mediate the flavonoid's *in vivo* effects. However, we found variations in the relative efficacy of 2'MeO6MF (2'MeO6MF-elicited current responses normalised to the maximal GABA response) at  $\alpha 2\beta 3\gamma 2L$  GABA<sub>A</sub>Rs due to the presence of mixed receptor populations. To understand which receptor subpopulation(s) underlie the variations observed, we conducted a systematic investigation of 2'MeO6MF activity at all receptor combinations that could theoretically form ( $\alpha 2, \beta 3, \gamma 2L, \alpha 2\beta 3, \alpha 2\gamma 2L, \beta 3\gamma 2L$  and  $\alpha 2\beta 3\gamma 2L$ ) in *Xenopus* oocytes using the two-electrode voltage clamp technique. We found that 2'MeO6MF activated non- $\alpha$ -containing  $\beta 3\gamma 2L$  receptors. In an attempt to establish the optimal conditions to express a uniform population of these receptors, we found that varying the relative amounts of  $\beta 3:\gamma 2L$  subunit mRNAs resulted in differences in the level of constitutive activity, the GABA concentration-response relationships, and the relative efficacy of 2'MeO6MF activation. Like 2'MeO6MF, general anaesthetics such as etomidate and propofol also showed distinct levels of relative efficacy across different injection ratios. Based on these results, we infer that  $\beta 3\gamma 2L$  receptors may form with different subunit stoichiometries, resulting in the complex pharmacology observed across different injection ratios. Moreover, the discovery that GABA and etomidate have direct actions at the  $\alpha$ -lacking  $\beta 3\gamma 2L$  receptors raises questions about the structural requirements for their respective binding sites at GABA<sub>A</sub>Rs.

## Introduction

$\gamma$ -aminobutyric acid type A receptors (GABA<sub>A</sub>Rs) are a ubiquitous class of inhibitory receptors with high physiological significance in the central nervous system (CNS). Structurally, GABA<sub>A</sub>Rs are made up of five pseudo-symmetrically arranged subunits surrounding a central anion-conducting pore. While a vast array of receptor combinations could theoretically form from the large number of known GABA<sub>A</sub>R subunits ( $\alpha 1-6$ ,  $\beta 1-3$ ,  $\gamma 1-3$ ,  $\delta$ ,  $\epsilon$ ,  $\theta$  and  $\pi$ ), most native GABA<sub>A</sub>R subtypes are made up of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits, and to a smaller extent  $\alpha$ ,  $\beta$  and  $\delta$  [1]. Receptors of different subunit composition have distinct distribution patterns, physiological roles, as well as electrophysiological and pharmacological properties [2–5].

GABA<sub>A</sub>Rs are the site of action for many clinically-relevant chemicals such as benzodiazepines, barbiturates and anaesthetics. These drugs typically bind to different interfaces between two neighbouring subunits that form the principal (+) and complementary (–) components of their binding sites. In the most common GABA<sub>A</sub>R isoform  $\alpha\beta\gamma$  with a stoichiometry of  $2\alpha:2\beta:1\gamma$  and a counter-clockwise arrangement of  $\gamma$ - $\beta$ - $\alpha$ - $\beta$ - $\alpha$  when viewed from the synaptic cleft [6–8], the extracellular domain of the  $\beta+\alpha$ – and the  $\alpha+\gamma$ – interfaces are known to harbour the GABA and benzodiazepine binding sites respectively [9–11]. In contrast, general anaesthetics such as etomidate and propofol have been shown to bind to the transmembrane domain of the  $\beta+\alpha$ – interface [11–13]. High prominence has been given to the  $\beta+\alpha$ – and  $\alpha+\gamma$ – interfaces due to the successful clinical utilisation of GABA<sub>A</sub>R modulators that bind to these interfaces. The pharmaceutical potential of the remaining  $\alpha+\beta$ – and  $\gamma+\beta$ – interfaces is also an area of active research [14–18].

Heterologously expressing a single defined GABA<sub>A</sub>R subtype to evaluate the structure-activity relationship of lead compounds has become an indispensable component in drug discovery. However, mixed receptor populations are a common problem that contributes to variability between pharmacological studies. For instance, the measurement of diazepam enhancement at  $\alpha\beta\gamma$  receptors has been shown to vary when contaminated with the diazepam-insensitive  $\alpha\beta$  receptors [19, 20]. Besides that, the binary  $\alpha\beta$  receptors have been shown to assemble with two different stoichiometries ( $2\alpha:3\beta$  and  $3\alpha:2\beta$ ), but the functional significance of this finding is not understood [6, 21, 22]. There have also been several independent reports of the robust  $\beta\gamma$  receptor expression and their responsiveness to GABA and various ligands, including diazepam, implicating binding sites for these ligands other than the traditional  $\beta+\alpha$ – and  $\alpha+\gamma$ – interfaces [23–29]. However, the impact of  $\beta\gamma$  receptors on the pharmacological studies conducted on  $\alpha\beta\gamma$  receptors, their potential as a tool receptor to interrogate GABA<sub>A</sub>R pharmacology, as well as their *in vivo* existence have not been explored.

We have previously reported that the synthetic flavonoid 2'-methoxy-6-methylflavone (2'MeO6MF) induced anxiolysis in mouse behavioural studies [30]. In single-channel recording experiments, 2'MeO6MF was found to directly activate native  $\gamma 2$ -containing GABA<sub>A</sub>Rs found in rat hippocampal neurons in the absence of GABA. Electrophysiological studies conducted across 18 different GABA<sub>A</sub>R subtypes expressed in *Xenopus* oocytes identified  $\alpha 2\beta 2/3\gamma 2L$  GABA<sub>A</sub>Rs as the targets for the direct activation of 2'MeO6MF. However, the activity of 2'MeO6MF at  $\beta\gamma$  receptors was not explored. Experimental evidence from electrophysiological and behavioural studies supports a non-benzodiazepine mechanism for 2'MeO6MF effects. Mutational studies found that the N265 residue on the  $\beta 2/3$  subunits, a key determinant of etomidate's sensitivity [31–33] is crucial for 2'MeO6MF activity, suggesting that 2'MeO6MF and etomidate may share a similar mechanism of action.

In this study, we found variations in 2'MeO6MF relative efficacy at  $\alpha 2\beta 3\gamma 2L$  receptors, which we associated with the presence of mixed receptor populations. We systematically expressed all possible receptor combinations in *Xenopus* oocytes using  $\alpha 2$ ,  $\beta 3$  and  $\gamma 2L$  subunits

and found that the non- $\alpha$ -containing  $\beta 3+\gamma 2L$  combination formed robust GABA-gated receptors which were also activated by 2'MeO6MF. Varying the mRNA injection ratio of  $\beta 3$  and  $\gamma 2L$  subunits resulted in unique pharmacological profiles for GABA, 2'MeO6MF, etomidate and propofol, most likely due to stoichiometric differences in the receptors being expressed. The  $\alpha$  subunit does not appear to be strictly needed for receptor function as demonstrated by the ability of GABA and etomidate to activate  $\beta 3\gamma 2L$  receptors. We infer from these findings that the  $\beta$  subunit, which bears the principal (+) components of the respective binding sites dominates ligand binding interactions, and the identity of the complementary (-) subunit ( $\alpha$ ,  $\beta$  or  $\gamma$ ) is well tolerated.

## Materials and Methods

### Reagents

GABA, flumazenil, zinc chloride ( $ZnCl_2$ ), DMSO and all buffer ingredients mentioned in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) while diazepam was purchased from Apin Chemicals LTD (Abingdon, Oxon, UK). Etomidate hydrochloride (HCl) was synthesised following protocols previously described in Janssen *et al.* [34] and Janssen *et al.* [35]. The synthesis of 2'MeO6MF was previously described in Karim *et al.* [30].

### GABA<sub>A</sub>R subunit constructs

Human complementary DNA (cDNA) of GABA<sub>A</sub>R  $\alpha 2$  subunit (vector: pCMV6-XL5) was purchased from OriGene Technologies (Rockville, MD, USA), and the human  $\beta 3$  (vector: pGEMHE) and  $\gamma 2L$  (vector: pCDM8) subunits were generous gifts from Dr. Bjarke Ebert (H. Lundbeck A/S, Valby, Denmark) and Dr. Paul Whiting (Merck, Sharp and Dohme Research Laboratories, Harlow, Essex, UK) respectively. The identity of the subunits was confirmed with DNA sequencing (Australian Genome Research Facility, Westmead, NSW, Australia). Plasmid vectors with the cDNA incorporated were linearised using appropriate restriction endonucleases ( $\alpha 2$ : SmaI;  $\beta 3$ : NheI;  $\gamma 2L$ : NotI). Capped RNA transcripts were synthesised from linearised plasmids using the mMessage mMachine T7 transcript kit from Ambion (Austin, TX, USA). Purified messenger RNA (mRNA) was dissolved in nuclease-free water (Ambion, Austin, TX, USA), and its quality was assessed using 1% agarose gel electrophoresis. The concentration of each subunit mRNA was measured using Nanodrop ND-100 UV-Vis spectrophotometer (Thermo Fisher Scientific, North Ryde, NSW, Australia).

### *Xenopus* oocytes isolation and preparation

Mature female *Xenopus laevis* frogs were anaesthetised with 0.17% tricaine (buffered with 0.06% sodium bicarbonate) for 15 minutes, after which the loss of righting reflex was confirmed before transferring on to ice where surgeries were performed. A small (1–2 cm) abdominal incision was made through both the skin and muscle layer with surgical knives. Ovary lobes were removed with a pair of forceps, and kept in oocyte releasing 2 (OR2) buffer (82.5 mM NaCl, 2 mM KCl, 1 mM  $MgCl_2$ , 5 mM HEPES hemisodium; pH 7.4). The skin and muscle layer were sutured separately, and frogs were allowed to recover for six months before they were reselected for surgeries. A total of five recoverable surgeries were allowed on each frog, before a terminal surgery was performed, in which a lethal dose of tricaine (0.5%) was used. All the procedures involved in the use of *Xenopus laevis* frogs were approved by the Animal Ethics Committee of the University of Sydney (Reference number: 2013/5915). Removed ovary lobes were divided into small sections and treated with 2 mg/mL Collagenase A (Boehringer Mannheim, Indianapolis, IN, USA) in OR2 buffer for at least an hour to defolliculate the

oocytes. The released oocytes were then rinsed in ND96 storage solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 5 mM HEPES hemisodium, 2.5 mM pyruvate, 0.5 mM theophylline; pH 7.4). Healthy-looking stage V–VI oocytes were isolated and kept in ND96 storage solution at 18°C until ready for injection.

## Electrophysiological recording of recombinant GABA<sub>A</sub>Rs in *Xenopus* oocytes

Various combinations of  $\alpha 2$ ,  $\beta 3$  and  $\gamma 2L$  subunit mRNAs were mixed to different ratios as stated in the text, and diluted accordingly to a concentration of 5 ng/ $\mu$ L (unless otherwise stated). The mRNA mixture was injected into the cytoplasm of oocytes (50.6 nL/injection) using a Nanoject device (Drummond Scientific, Broomali, PA, USA). Injected oocytes were stored in ND96 storage solution supplemented with 50  $\mu$ g/mL gentamycin and tetracycline at 18°C. Two-electrode voltage clamp recordings were performed on oocytes 2–4 days post-injection at room temperature (20–25°C) using a GeneClamp 500B amplifier (ADInstruments, Sydney, NSW, Australia) or a Warner OC-725C Oocyte Clamp (Warner Instrument Corp, Hamden, CT, USA). All experiments were conducted at a holding potential of –60 mV, and data were acquired with a PowerLab 2/25, and LabChart (version 5.0.2) software (ADInstruments, Sydney, NSW, Australia). The recording microelectrodes (0.2–1.1 M $\Omega$ ) were generated from capillary glass (Harvard Apparatus, Holliston, MA, USA) using a single-stage glass microelectrode puller (Narishige, Tokyo, Japan), and were filled with 3 M KCl solution. The perfusion system was built using a series of polycarbonate, high-density polyethylene two-way stopcocks (Qosina, Edgewood, NY, USA), connected through tubing to the recording chamber (100  $\mu$ L), where the oocyte was continuously perfused with ND96 recording solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 5 mM HEPES hemisodium; pH 7.4) at an approximate rate of 5 mL/min.

GABA, etomidate and ZnCl<sub>2</sub> were dissolved in ND96 recording solution, whereas 2'MeO6MF, diazepam and flumazenil were dissolved in DMSO to make up stock solutions of desired concentrations. GABA stock solutions were prepared fresh on the day of experiments. When working with chemicals dissolved in DMSO, all perfusates were standardised to contain 0.8% DMSO, which did not produce any alteration in the recordings. Perfusates containing these chemicals were made up fresh before each application. The concentration-response curves of agonists were obtained by exposing oocytes to increasing concentrations of agonists until the currents peaked. Maximal GABA current responses (usually 3 mM, unless otherwise stated) were used as controls to allow for comparisons between different oocytes. Depending on the concentration and the solubility of the chemicals applied on oocytes, the wash-out periods ranged from 3 to 15 minutes to allow time for receptors to recover from desensitisation. The modulatory effect of 2'MeO6MF was also investigated by co-applying a low concentration of GABA (EC<sub>3–10</sub>) with a high concentration of 2'MeO6MF (100 or 300  $\mu$ M). No enhancement of GABA current was detected at  $\alpha 2\beta 3\gamma 2L$  (3:1:3),  $\alpha 2\beta 3$  (20:1),  $\beta 3\gamma 2L$  (1:15) and (1:100) receptors (data not shown). All experiments were performed using at least two different batches of oocytes.

## Data analysis

Prism (version 5.04; GraphPad Software, La Jolla, CA, USA) was used for data analysis. Raw data from GABA concentration-response experiments were fitted to either a monophasic (1) or biphasic (2) Hill equation, and the fitted maximal values from the preferred model (Extra

sum-of-squares  $F$  test) were used for normalisation of each dataset.

$$I = \frac{I_{\max}}{1 + 10^{(\log EC_{50} - [A])n_H}} \quad (1)$$

$$I = \frac{I_{\max} \times \text{Frac}}{1 + 10^{(\log EC_{50L1} - [A])n_{HL1}}} + \frac{I_{\max} \times (1 - \text{Frac})}{1 + 10^{(\log EC_{50L2} - [A])n_{HL2}}} \quad (2)$$

Concentration-response curves of other agonists such as 2'MeO6MF and etomidate were constructed in a similar manner, except the data were normalised to the 3 mM GABA responses, unless otherwise stated. In these equations,  $I_{\max}$  represents the maximal agonist response,  $[A]$  represents the agonist concentration,  $EC_{50}$  represents the agonist concentration required to activate 50% of the maximal response (there are two  $EC_{50}$ s for a biphasic curve),  $n_H$  represents the Hill slope of the fitted curve (there are two Hill slopes for a biphasic curve), and  $\text{Frac}$  represents the proportion of the maximal response mediated by the more potent component in a biphasic curve. In all cases, we constrained the bottom to 0, and when fitting data to the biphasic model, the Hill slopes were constrained to 1. Statistical comparisons of results for different injection ratios of  $\beta 3\gamma 2L$  receptors were performed using ANOVA with Tukey's *post-hoc* test. When comparing results from different injection ratios of  $\beta 3\gamma 2L$  receptors with  $\alpha 2\beta 3\gamma 2L$  receptors, the ANOVA Dunnett's test was used. Pairwise comparisons were performed using Student's  $t$  test. Statistical significance was attained at  $p < 0.05$ .

## Results

### Variations in the relative efficacy of 2'MeO6MF activation at $\alpha 2\beta 3\gamma 2L$ GABA<sub>A</sub>Rs

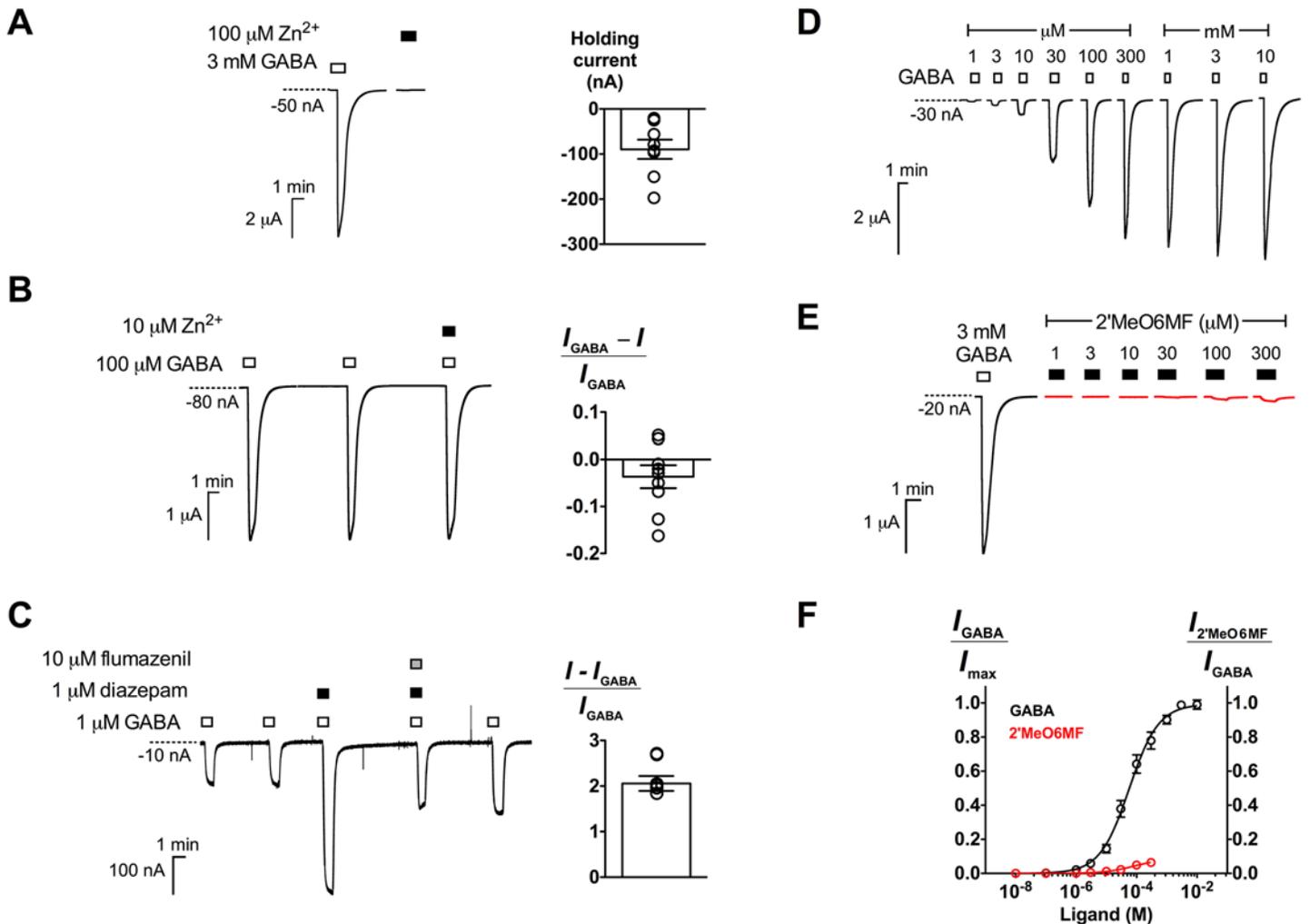
2'MeO6MF has been previously described as a full agonist at  $\alpha 2\beta 3\gamma 2L$  GABA<sub>A</sub>Rs expressed at a 1:1:10 injection ratio in *Xenopus* oocytes [30]. We expressed  $\alpha 2\beta 3\gamma 2L$  receptors at a 3:1:3 injection ratio and recorded pharmacological responses to GABA,  $Zn^{2+}$ , diazepam (1  $\mu M$ ) and flumazenil (10  $\mu M$ ) that were consistent with those previously reported (Fig 1 and Table 1) [36–38]. In contrast to what was reported by Karim *et al.* (2012) [30], we found that 300  $\mu M$  2'MeO6MF only activated  $\alpha 2\beta 3\gamma 2L$  receptors  $8.2 \pm 3.6\%$  of the 3 mM GABA response ( $n = 5$ ; Fig 1E and 1F). The  $EC_{50}$  of 2'MeO6MF was estimated to be approximately 74  $\mu M$  (95% CI: 6.4–850  $\mu M$ ). We hypothesised that the variations in the relative efficacy of 2'MeO6MF measured at  $\alpha 2\beta 3\gamma 2L$  receptors expressed at different ratios may be due to the presence of mixed receptor populations.

### 2'MeO6MF does not activate binary $\alpha 2\beta 3$ or homomeric $\alpha 2$ , $\beta 3$ and $\gamma 2L$ GABA<sub>A</sub>Rs

The presence of  $\alpha\beta$  receptors when expressing  $\alpha\beta\gamma$  receptors has been shown to result in sub-maximal measurement of diazepam modulation [19, 20] as these receptors lack the  $\alpha+\gamma$  interface where diazepam binds. To determine if this was the cause for differences in 2'MeO6MF relative efficacy at  $\alpha 2\beta 3\gamma 2L$  receptors, we expressed  $\alpha 2:\beta 3$  subunit mRNAs at a 20:1 ratio to prevent  $\beta 3$  homomeric receptors from forming, and found that 2'MeO6MF (100  $\mu M$ ) had negligible activity at these receptors, eliciting currents only  $1.7 \pm 0.35\%$  of the 3 mM GABA responses ( $n = 5$ ; Fig 2A). Therefore,  $\alpha 2\beta 3$  receptors are unlikely to contribute to the much higher 2'MeO6MF relative efficacy previously reported at  $\alpha 2\beta 3\gamma 2L$  GABA<sub>A</sub>Rs.

To determine if homomeric GABA<sub>A</sub>Rs are activated by 2'MeO6MF, we injected  $\alpha 2$ ,  $\beta 3$  and  $\gamma 2L$  mRNAs alone and examined the effect of 2'MeO6MF at these receptors. We did not detect

$\alpha 2\beta 3\gamma 2L$  (3:1:3)



**Fig 1. Characterisation of  $\alpha 2\beta 3\gamma 2L$  GABA<sub>A</sub>Rs expressed at a 3:1:3 injection ratio.** (A) *Left panel*, Representative traces of  $\alpha 2\beta 3\gamma 2L$  (3:1:3) GABA<sub>A</sub>Rs responses to 3 mM GABA and 100  $\mu M$  Zn<sup>2+</sup> alone. *Right panel*, Mean holding current of oocytes expressing  $\alpha 2\beta 3\gamma 2L$  (3:1:3) GABA<sub>A</sub>Rs ( $-93 \pm 19$  nA;  $n = 8$ ). (B) *Left panel*, Continuous traces demonstrating two consecutive applications of control (100  $\mu M$  GABA) followed by the co-application of 10  $\mu M$  Zn<sup>2+</sup> with control. *Right panel*, Modulation of 100  $\mu M$  GABA responses by 10  $\mu M$  Zn<sup>2+</sup> ( $n = 9$ ). (C) *Left panel*, Continuous traces demonstrating two consecutive applications of control (1  $\mu M$  GABA) followed by the co-application of 1  $\mu M$  diazepam with control; 1  $\mu M$  diazepam and 10  $\mu M$  flumazenil with control; and control. *Right panel*, Potentiation by 1  $\mu M$  diazepam of 1  $\mu M$  GABA responses ( $n = 6$ ). Representative traces demonstrating (D) GABA current responses from 1  $\mu M$  to 10 mM and (E) 2'MeO6MF's direct activation from 1 to 300  $\mu M$  (red) in comparison to 3 mM GABA response. Concentration-response curves of GABA (black;  $n = 6$ ) and 2'MeO6MF (red;  $n = 5$ ) are shown in (F). Data are presented as mean  $\pm$  SEM. Bars indicate durations of drug application. The holding current values are represented by the dotted lines.

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function with 60 mM GABA and 100  $\mu M$  2'MeO6MF in *Xenopus* oocytes injected with  $\alpha 2$  mRNA ( $n = 5$ ; Table 1).  $\gamma 2L$  homomeric receptors showed Zn<sup>2+</sup>-sensitive constitutive activity, but were not responsive to 60 mM GABA and 100  $\mu M$  2'MeO6MF ( $n = 5$ ; Fig 2B and Table 1). Consistent with previous findings [39, 40], the injection of  $\beta 3$  subunit mRNA resulted in the expression of constitutively active receptors, which are activated by high concentrations of GABA (Fig 2C and Table 1). In contrast to the direct activation observed at  $\alpha 2\beta 3\gamma 2L$  GABA<sub>A</sub>Rs, 2'MeO6MF inhibited the constitutive activity of  $\beta 3$  homomeric receptors in a

**Table 1. Characteristics of receptors expressed in various combinations of  $\alpha 2$ ,  $\beta 3$  and  $\gamma 2L$  GABA<sub>A</sub>R subunits.**

Subunit combination	Ratio	mRNA amount (ng/oocyte)	Expression level (nA) <sup>a</sup>	Holding current (nA)	n	2'MeO6MF activity	n
$\alpha 2$	–	28–35	No current	-20 ± 7.5	8	No current	5
$\beta 3$	–	5	290 ± 67	-1000 ± 420	6	Inverse agonism	5
$\gamma 2L$	–	20–35	No current	-370 ± 41	7	No current	5
$\alpha 2\beta 3$	20:1	5	1100 ± 220	-30 ± 7.0	7	Insignificant activation	5
$\alpha 2\gamma 2L$	1:1	5	No current	-42 ± 16	4	No current	4
$\beta 3\gamma 2L$	1:1	5	110 ± 34	-470 ± 88	4	Inverse agonism	4
	1:5	5	270 ± 80	-220 ± 60	5	Inverse agonism/activation	5
	1:10	5	310 ± 61	-150 ± 13	8	Inverse agonism/activation	8
	1:15	5	490 ± 47	-260 ± 40	30	Activation	30
	1:20	5	860 ± 87	-180 ± 31	49	Activation	49
	1:50	5	1000 ± 160	-28 ± 6.0	15	Activation	15
$\alpha 2\beta 3\gamma 2L$	3:1:3	5	4300 ± 940	-93 ± 19	8	Activation	5

<sup>a</sup> Expression level is expressed as the current responses ± SEM (nA) elicited by 3 mM GABA, except for homomeric and  $\alpha 2\gamma 2L$  receptors (60 mM GABA).

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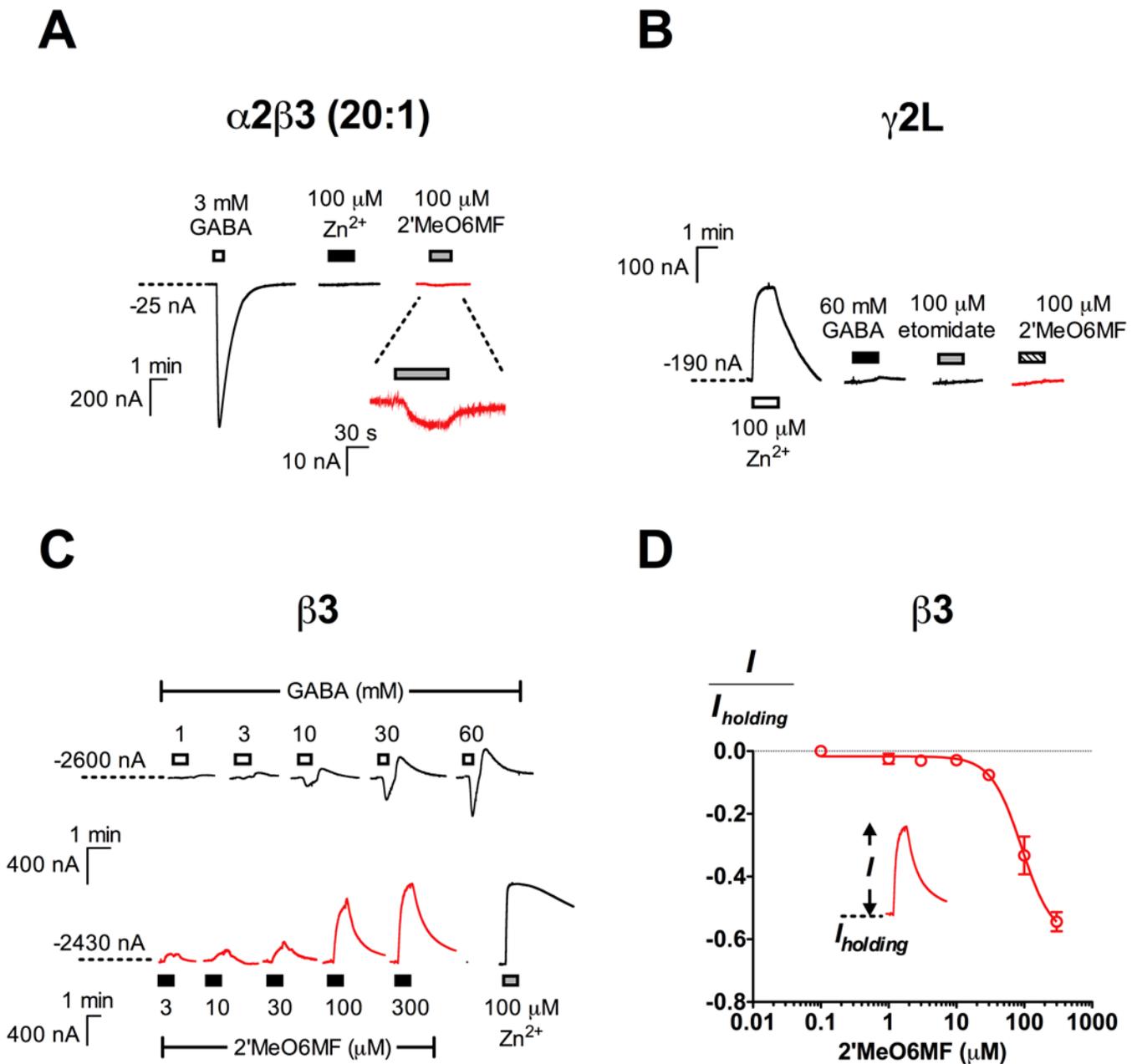
concentration-dependent manner, with a maximal inhibition of  $54 \pm 3.0\%$  (normalised against oocyte holding current) measured at 300  $\mu M$  2'MeO6MF ( $n = 5$ ; Fig 2C and 2D). The lack of activation by 2'MeO6MF at homomeric receptors demonstrates that these receptors are unlikely to be the cause for the variations observed.

### 2'MeO6MF activates $\beta 3\gamma 2L$ GABA<sub>A</sub>Rs with variable efficacy

We then determined whether binary  $\alpha 2\gamma 2L$  or  $\beta 3\gamma 2L$  receptors are activated by 2'MeO6MF. Binary  $\alpha 2\gamma 2L$  receptors showed no function to 60 mM GABA, 100  $\mu M$  Zn<sup>2+</sup> and 100  $\mu M$  2'MeO6MF ( $n = 4$ ; Table 1). In contrast, injection of  $\beta 3$  and  $\gamma 2L$  mRNAs at a 1:20 ratio to prevent the expression of  $\beta 3$  homomers yielded receptors that responded to GABA. 2'MeO6MF efficaciously activated these receptors, with 100  $\mu M$  eliciting currents approximately  $34 \pm 4.0\%$  of the 3 mM GABA responses ( $n = 49$ ; Fig 3). Notably, there was a high variability in the relative efficacy of 2'MeO6MF, ranging from as low as 1.0 to 120% of the 3 mM GABA responses. While the standard error of mean efficacy was only 4.0%, the standard deviation (SD) remained high (31%). This variability was observed both between different batches of oocytes and within the same batch. The substantial variability in the relative efficacy of 2'MeO6MF suggests that the 1:20 ratio was not optimal for the uniform expression of  $\beta 3\gamma 2L$  receptors.

To establish the optimal ratio(s) for the uniform expression of  $\beta 3\gamma 2L$  GABA<sub>A</sub>Rs, we varied the  $\beta 3:\gamma 2L$  mRNAs ratio (1:1, 1:5, 1:10, 1:15, 1:50 and 1:100) while keeping the total amount of mRNA constant at 5 ng in each oocyte. We then measured the relative efficacy of 100  $\mu M$  2'MeO6MF and compared it across these receptors. At 1:1 ratio,  $\beta 3\gamma 2L$  receptors were constitutively active and 2'MeO6MF acted as an inverse agonist (Fig 3A). This is likely due to the presence of a substantial amount of  $\beta 3$  homomer subpopulation. At 1:5 and 1:10 ratios, where the relative amounts of  $\gamma 2L$  mRNA were greater than  $\beta 3$  mRNA, 2'MeO6MF displayed a mixed inverse agonist and agonist effect (Fig 3A), indicating that the pharmacological responses of 2'MeO6MF observed at these ratios were still confounded by  $\beta 3$  homomers.

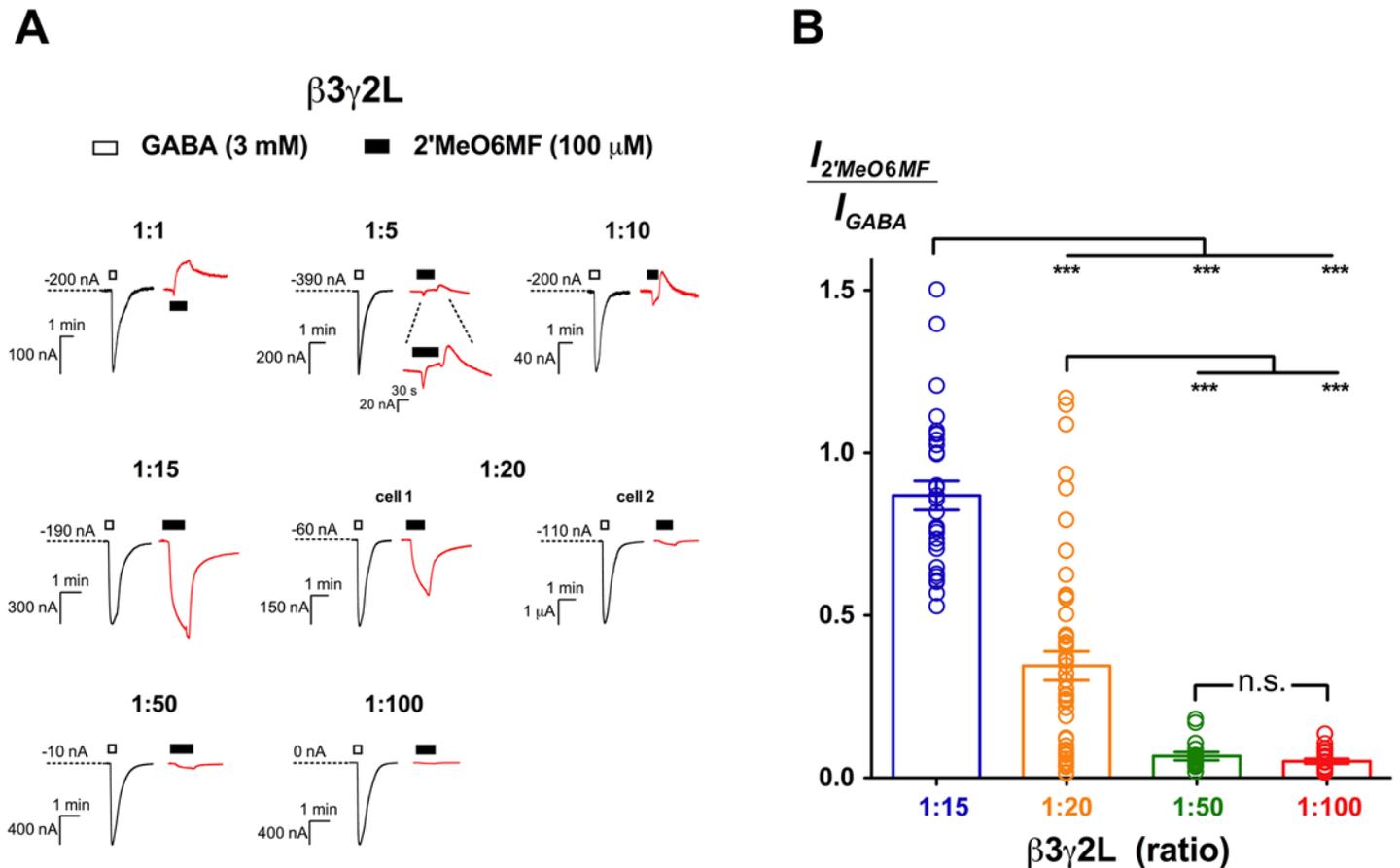
We increased the injection ratio to 1:15, and consistently observed activation with 100  $\mu M$  2'MeO6MF (Fig 3A). The 2'MeO6MF-elicited currents were  $87 \pm 4.5\%$  of the 3 mM GABA current responses ( $n = 30$ ), which was significantly higher than that at 1:20 ratio ( $p \leq 0.001$ ; Tukey's test; Fig 3B). While the low-efficacy component of 2'MeO6MF was not observed at



**Fig 2. Subunit combinations with detectable function but were not activated by 2'MeO6MF.** (A) Representative traces illustrating the robust response elicited by 3 mM GABA, and the lack of activity of 100  $\mu M$   $Zn^{2+}$  and 100  $\mu M$  2'MeO6MF at  $\alpha 2\beta 3$  (20:1) GABA<sub>A</sub>Rs ( $n = 6$ ). (B) The injection of  $\gamma 2L$  mRNA at high amounts (28–35 ng/oocyte) resulted in constitutively active channels which were inhibited by 100  $\mu M$   $Zn^{2+}$ , but were not sensitive to 60 mM GABA, 100  $\mu M$  etomidate and 100  $\mu M$  2'MeO6MF ( $n = 5$ ). (C) The injection of  $\beta 3$  mRNA (5 ng/oocyte) resulted in the formation of functional receptors. Representative traces of  $\beta 3$  homomeric receptors responses to 1–60 mM GABA (top); 3–300  $\mu M$  of 2'MeO6MF and 100  $\mu M$   $Zn^{2+}$  (bottom). (D) 2'MeO6MF concentration-response curve for  $\beta 3$  homomeric receptors ( $n = 5$ ). The efficacy of 2'MeO6MF as an inverse agonist is expressed as a fraction of the inhibited spontaneous current ( $I$ ) normalised against the holding current ( $I_{holding}$ ). Data are presented as mean  $\pm$  SEM. Bars indicate durations of drug application. The holding current values are represented by the dotted lines.

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1:15 ratio, there was still considerable variability in the relative efficacy measured (SD = 25%). Increasing the injection ratios further to 1:50 and 1:100 abolished the high-efficacy component of 2'MeO6MF. 100  $\mu M$  2'MeO6MF showed efficacy around  $6.7 \pm 1.2\%$  ( $n = 15$ ) at 1:50 ratio



**Fig 3. 2'MeO6MF activity at  $\beta\gamma 2L$  GABA<sub>A</sub>Rs.** (A) 2'MeO6MF exhibits a complex spectrum of activity across  $\beta\gamma 2L$  GABA<sub>A</sub>Rs expressed at various injection ratios. Representative traces of 3 mM GABA (black) and 100  $\mu$ M 2'MeO6MF (red) are shown for each ratio. Bars indicate durations of drug application. The holding current values are represented by the dotted lines. (1:1;  $n = 7$ ), 2'MeO6MF inhibited the constitutive activity of receptors expressed. (1:5;  $n = 5$ ) and (1:10;  $n = 10$ ), 2'MeO6MF exhibited mixed agonist and inverse agonist activity. (1:15;  $n = 30$ ), 2'MeO6MF directly activated receptors expressed efficaciously. (1:20;  $n = 49$ ), 2'MeO6MF activated  $\beta\gamma 2L$  (1:20) receptors with variable efficacy. Sample traces of cell 1 and 2 were taken from a simultaneous experiment conducted on two different oocytes injected at the same time. (1:50;  $n = 15$ ) and (1:100;  $n = 21$ ), 2'MeO6MF showed activation with low efficacy at these receptors. (B) Mean efficacy of 100  $\mu$ M 2'MeO6MF direct activation at  $\beta\gamma 2L$  (1:15), (1:20), (1:50) and (1:100) GABA<sub>A</sub>Rs. Data are normalised to the 3 mM GABA response. The mean efficacy of 100  $\mu$ M 2'MeO6MF at various ratios was compared using Tukey's test, and the significance levels are indicated with n.s. (not significant) and \*\*\* ( $p \leq 0.001$ ).

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and  $5.1 \pm 0.72\%$  ( $n = 21$ ) at 1:100 ratio, which is significantly lower than at the 1:15 ratio ( $p \leq 0.001$ ; Tukey's test; Fig 3B). The relative efficacy of 2'MeO6MF measured at  $\alpha 2\beta\gamma 2L$  receptors was similar to that of  $\beta\gamma 2L$  receptors expressed at 1:50 and 1:100 ratios ( $p > 0.5$ ; Dunnett's test), but was significantly lower than 1:15 ( $p \leq 0.001$ ) and 1:20 ( $p \leq 0.5$ ) ratios.

The extensive variability in 2'MeO6MF relative efficacy measured at 1:20 ratio was likely to be the result of mixed stoichiometries being expressed. This is further supported by variability observed in the GABA concentration-response relationships at  $\beta\gamma 2L$  (1:20) receptors, in which out of 13 datasets, 6 were better fitted to a biphasic Hill equation, whereas a simpler monophasic model was preferred to describe the rest of the datasets (data not shown). Under our conditions, the 1:15, 1:50 and 1:100 ratios were most optimal to express the different stoichiometries of  $\beta\gamma 2L$  receptors, allowing us to measure 2'MeO6MF responses more reliably. As such, these ratios were chosen for detailed pharmacological characterisation.

## $\beta\gamma 2L$ GABA<sub>A</sub>Rs of different subunit stoichiometries vary in pharmacological properties

To obtain an understanding of the properties of different  $\beta\gamma 2L$  receptor stoichiometries, we assessed constitutive activity, 2'MeO6MF and GABA potencies of the receptors expressed at 1:15, 1:50 and 1:100 injection ratios. Oocytes expressing  $\beta\gamma 2L$  (1:15) receptors exhibited significantly larger, more negative holding currents ( $-260 \pm 40$  nA) in comparison to 1:50 ( $-28 \pm 6.0$  nA) and 1:100 ( $-15 \pm 5.0$  nA) ratios ( $p \leq 0.001$ ; Tukey's test; Fig 4A). The application of  $100 \mu\text{M Zn}^{2+}$  generated a reduction in inward current at  $\beta\gamma 2L$  (1:15) receptors (Fig 4B). In contrast,  $100 \mu\text{M Zn}^{2+}$  did not have any effect at  $\beta\gamma 2L$  (1:50) and (1:100) receptors.

Despite the difference in maximal efficacy (Fig 4C), 2'MeO6MF showed similar potency at all ratios ( $EC_{50} = 45 \mu\text{M}$  (1:15),  $62 \mu\text{M}$  (1:50),  $63 \mu\text{M}$  (1:100);  $p > 0.05$  for all  $\log EC_{50}$  comparisons; Tukey's test; Fig 4D and Table 2). The potency of 2'MeO6MF measured at  $\alpha 2\beta\gamma 2L$  receptors is also not significantly different from  $\beta\gamma 2L$  receptors at all three ratios ( $p > 0.05$  for all comparisons; Dunnett's test). We then measured the GABA potency, and found that  $\beta\gamma 2L$  (1:15) receptors displayed a monophasic GABA concentration-response curve with low potency and a shallow Hill slope ( $EC_{50} = 1.4$  mM;  $n_H = 0.54$ ;  $n = 6$ ; Fig 4E and 4F and Table 2). In contrast, the data of 1:50 and 1:100 ratios were significantly better fitted to a biphasic Hill equation than a monophasic model ( $p < 0.0001$  for both ratios; extra sum-of-squares  $F$  test; Fig 4E and 4F). At 1:50 ratio, GABA has a high-sensitivity component with an  $EC_{50}$  of  $63 \mu\text{M}$  and a low-sensitivity component with an  $EC_{50}$  of  $5.5$  mM. The mean  $EC_{50}$  values for  $\beta\gamma 2L$  (1:100) receptors were very similar to those of  $\beta\gamma 2L$  (1:50) receptors ( $EC_{50_1} = 64 \mu\text{M}$ ;  $EC_{50_2} = 4.0$  mM).  $\beta\gamma 2L$  GABA<sub>A</sub>Rs expressed at 1:50 and 1:100 ratios exhibit nearly identical properties (no statistical significant differences in all aspects;  $p > 0.05$ ), but are very different in comparison to the receptors expressed at 1:15 ratio.

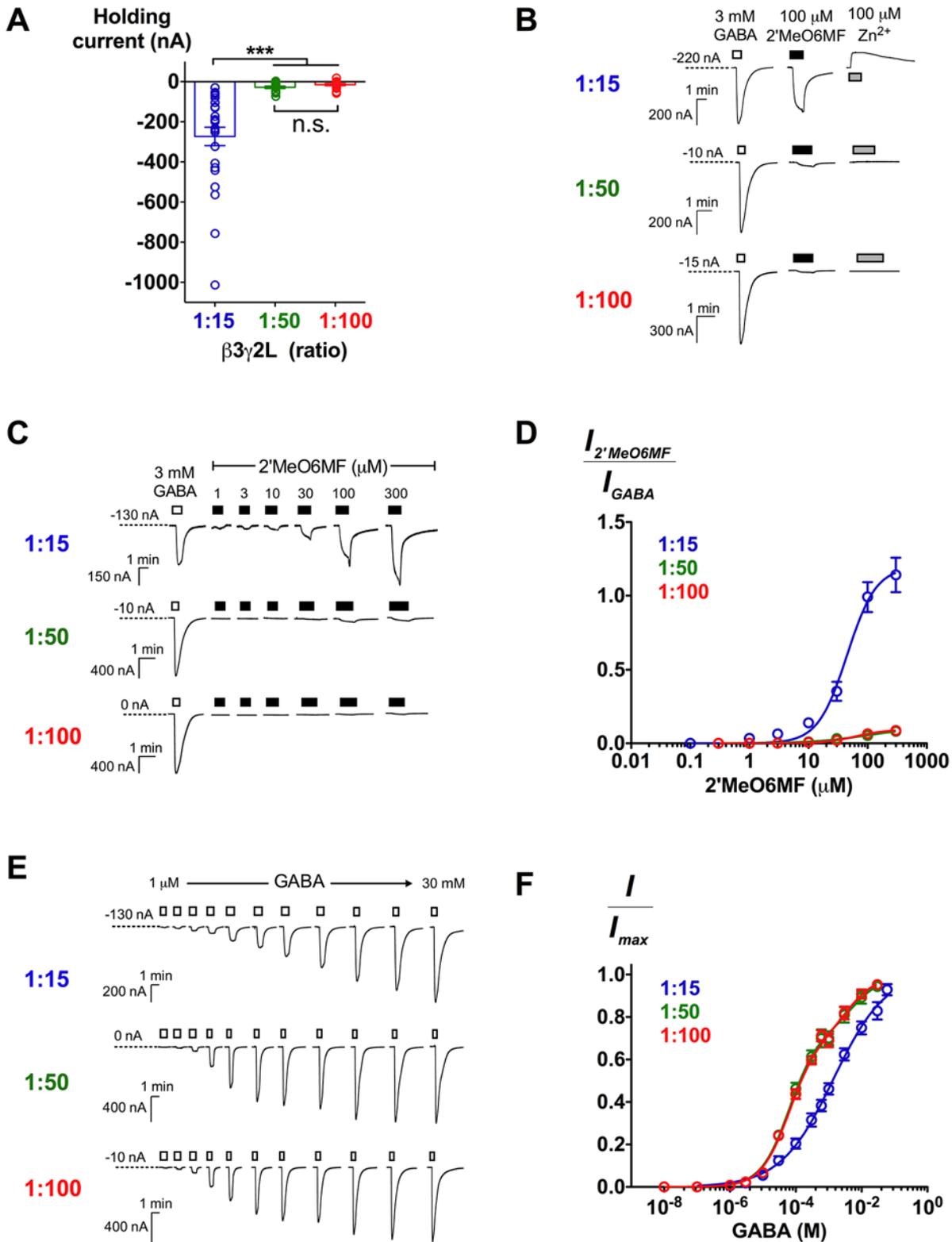
## Differential activation of $\beta\gamma 2L$ receptors with different subunit stoichiometries by general anaesthetics

2'MeO6MF activity has previously been shown to be affected by a well-established mutation that perturbs etomidate activity [30], suggesting that both ligands could share a common activation mechanism. Like 2'MeO6MF, etomidate did not activate homomeric  $\gamma 2L$  (Fig 2B) or  $\alpha 2\gamma 2L$  receptors ( $n = 4$ ; data not shown). However, etomidate activated  $\beta 3$  homomeric receptors efficaciously ( $E_{\text{max}} = 15 \pm 0.79$ , normalised against  $60$  mM GABA), with an  $EC_{50}$  of  $7.7 \mu\text{M}$  (95% CI:  $5.6$ – $11 \mu\text{M}$ ;  $n = 5$ ; Fig 5E and 5F), in contrast to 2'MeO6MF inhibition of the receptors' constitutive activity (Fig 2C).

We then evaluated etomidate at  $\beta\gamma 2L$  (1:15), (1:50) and (1:100) receptors. Etomidate directly activated  $\beta\gamma 2L$  receptors expressed at all three ratios, albeit with different relative efficacies (Fig 5A). At  $10 \mu\text{M}$ , etomidate directly activated  $\beta\gamma 2L$  (1:15) approximately  $12 \pm 1.1$  fold higher relative to  $3$  mM GABA ( $n = 7$ ), significantly greater than at 1:50 ( $0.46 \pm 0.10$ ;  $n = 6$ ) and 1:100 ( $0.27 \pm 0.05$ ;  $n = 7$ ) ratios ( $p \leq 0.001$ ; Tukey's test; Fig 5B). At  $\beta\gamma 2L$  (1:50) and (1:100) receptors, consistent with the almost identical GABA and 2'MeO6MF profiles, etomidate activated  $\beta\gamma 2L$  receptors at both ratios with no significant differences in potencies and maximal efficacies ( $p > 0.05$ ; unpaired  $t$  test; Fig 5C and Table 2). At 1:15 ratio, we could not construct a complete concentration-response curve as the current elicited by etomidate at concentrations above  $10 \mu\text{M}$  frequently exceeded the measuring range of the amplifier ( $> 10 \mu\text{A}$ ).

To investigate etomidate activity across the whole concentration range at  $\beta\gamma 2L$  (1:15) receptors, we conducted electrophysiological recording at  $-30$  mV. The same experiment was repeated at  $\beta\gamma 2L$  (1:100) receptors as a control. As expected from the voltage-dependent profile of etomidate [41], we observed reduced etomidate activation at  $-30$  mV. However,

## $\beta 3\gamma 2L$ (1:15, 1:50, 1:100)



**Fig 4. Characterisation of  $\beta 3\gamma 2L$  GABA<sub>A</sub>Rs expressed at 1:15, 1:50 and 1:100 ratios.** The level of constitutive activity is indicated by (A) holding current of injected oocytes and (B) the inhibition of baseline current by 100  $\mu M$  Zn<sup>2+</sup>. (A)  $\beta 3\gamma 2L$  GABA<sub>A</sub>Rs expressed at 1:15 ratio showed significantly larger holding current ( $-260 \pm 40$  nA;  $n = 30$ ) than at 1:50 ( $-28 \pm 6.0$  nA;  $n = 15$ ) and 1:100 ( $-15 \pm 5.0$  nA;  $n = 21$ ) ratios ( $p \leq 0.001$ ; Tukey's test). (B) Representative traces demonstrating current responses of 3 mM GABA, 100  $\mu M$  2'MeO6MF and 100  $\mu M$  Zn<sup>2+</sup>. At 1:15 ratio, receptors were sensitive to the inhibition of 100  $\mu M$  Zn<sup>2+</sup> (reduction in inward current;  $n = 10$ ). Zn<sup>2+</sup> did not have any effects at 1:50 ( $n = 7$ ) and 1:100 ( $n = 8$ ) ratios. (C) Representative traces demonstrating 2'MeO6MF's direct activation from 1 to 300  $\mu M$  in comparison to 3 mM GABA response. (D) 2'MeO6MF concentration-response curves of  $\beta 3\gamma 2L$  (1:15;  $n = 8$ ), (1:50;  $n = 7$ ) and (1:100;  $n = 6$ ) GABA<sub>A</sub>Rs. Data are normalised to the 3 mM GABA response. (E) Representative traces of GABA current responses from 1  $\mu M$  to 30 mM at  $\beta 3\gamma 2L$  (1:15), (1:50) and (1:100) GABA<sub>A</sub>Rs. (F) GABA concentration-response curves of  $\beta 3\gamma 2L$  (1:15;  $n = 6$ ), (1:50;  $n = 8$ ) and (1:100;  $n = 9$ ) GABA<sub>A</sub>Rs. Data are presented as mean  $\pm$  SEM. Bars indicate durations of drug application. The holding current values are represented by the dotted lines.

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etomidate still exhibited significantly larger relative efficacy at 1:15 ( $9.9 \pm 0.84$ ) than 1:100 ( $2.8 \pm 0.50$ ) ratio ( $p \leq 0.01$ ; unpaired  $t$  test; Fig 5D; Table 2). Etomidate also showed a modest,

**Table 2. GABA, 2'MeO6MF, etomidate and propofol concentration-response curve parameters at  $\beta 3\gamma 2L$  (1:15), (1:50) and (1:100) GABA<sub>A</sub>Rs derived from curve-fitting procedures.**

Ligands		$\beta 3\gamma 2L$ (ratio) <sup>a</sup>		
		1:15	1:50	1:100
GABA <sup>b</sup>	$I_{max} \pm SEM^c$	780 $\pm$ 220 nA	1000 $\pm$ 250 nA	1000 $\pm$ 97 nA
	EC <sub>50_1</sub> (95% CI)	1.4 (0.87–2.2) mM	63 (47–84) $\mu M$	64 (49–83) $\mu M$
	EC <sub>50_2</sub> (95% CI)	–	5.5 (1.3–23) mM	4.0 (1.4–11) mM
	$n_{H_1} \pm SEM$	0.54 $\pm$ 0.040	1.0	1.0
	$n_{H_2}$	–	1.0	1.0
	Frac (95% CI)	–	0.74 (0.67–0.80)	0.71 (0.65–0.77)
	$n$	6	8	9
2'MeO6MF	$E_{max} \pm SEM^d$	1.2 $\pm$ 0.10	0.086 $\pm$ 0.018	0.10 $\pm$ 0.020
	EC <sub>50</sub> (95% CI)	45 (31–64) $\mu M$	62 (22–172) $\mu M$	63 (27–143) $\mu M$
	$n_H \pm SEM$	1.8 $\pm$ 0.42	1.4 $\pm$ 0.88	1.6 $\pm$ 0.76
	$n$	8	7	6
Etomidate (-60 mV)	$E_{max} \pm SEM^e$	20 $\pm$ 1.8	5.5 $\pm$ 0.80	3.2 $\pm$ 0.40
	EC <sub>50</sub> (95% CI)	8.7 (6.7–11) $\mu M$	55 (27–110) $\mu M$	43 (25–76) $\mu M$
	$n_H \pm SEM$	2.0 $\pm$ 0.32	1.3 $\pm$ 0.37	1.5 $\pm$ 0.46
	$n$	5	6	7
Etomidate (-30 mV)	$E_{max} \pm SEM^f$	9.9 $\pm$ 0.84	N.D. <sup>g</sup>	2.8 $\pm$ 0.50
	EC <sub>50</sub> (95% CI)	19 (11–31) $\mu M$		60 (19–190) $\mu M$
	$n_H \pm SEM$	1.3 $\pm$ 0.33		1.2 $\pm$ 0.50
	$n$	4		5
Propofol	$E_{max} \pm SEM^h$	5.7 $\pm$ 0.55	N.D. <sup>g</sup>	0.64 $\pm$ 0.23
	EC <sub>50</sub> (95% CI)	85 (33–220) $\mu M$		74 (33–170) $\mu M$
	$n_H \pm SEM$	1.4 $\pm$ 0.50		2.0 $\pm$ 1.2
	$n$	4		4

<sup>a</sup> The total amount of mRNA injected per oocyte was adjusted to be approximately 5 ng for each ratio.

<sup>b</sup> Data of 1:15 ratio were better fitted to a monophasic model. Data of 1:50 and 1:100 ratios were significantly better fitted to a biphasic model ( $p < 0.0001$  for both ratios; extra sum-of-squares  $F$  test). Concentration-response curves are shown in Fig 4F.

<sup>c</sup> Current amplitude elicited by 30 mM GABA.

<sup>d</sup> 300  $\mu M$  2'MeO6MF-elicited current normalised against 3 mM GABA response.

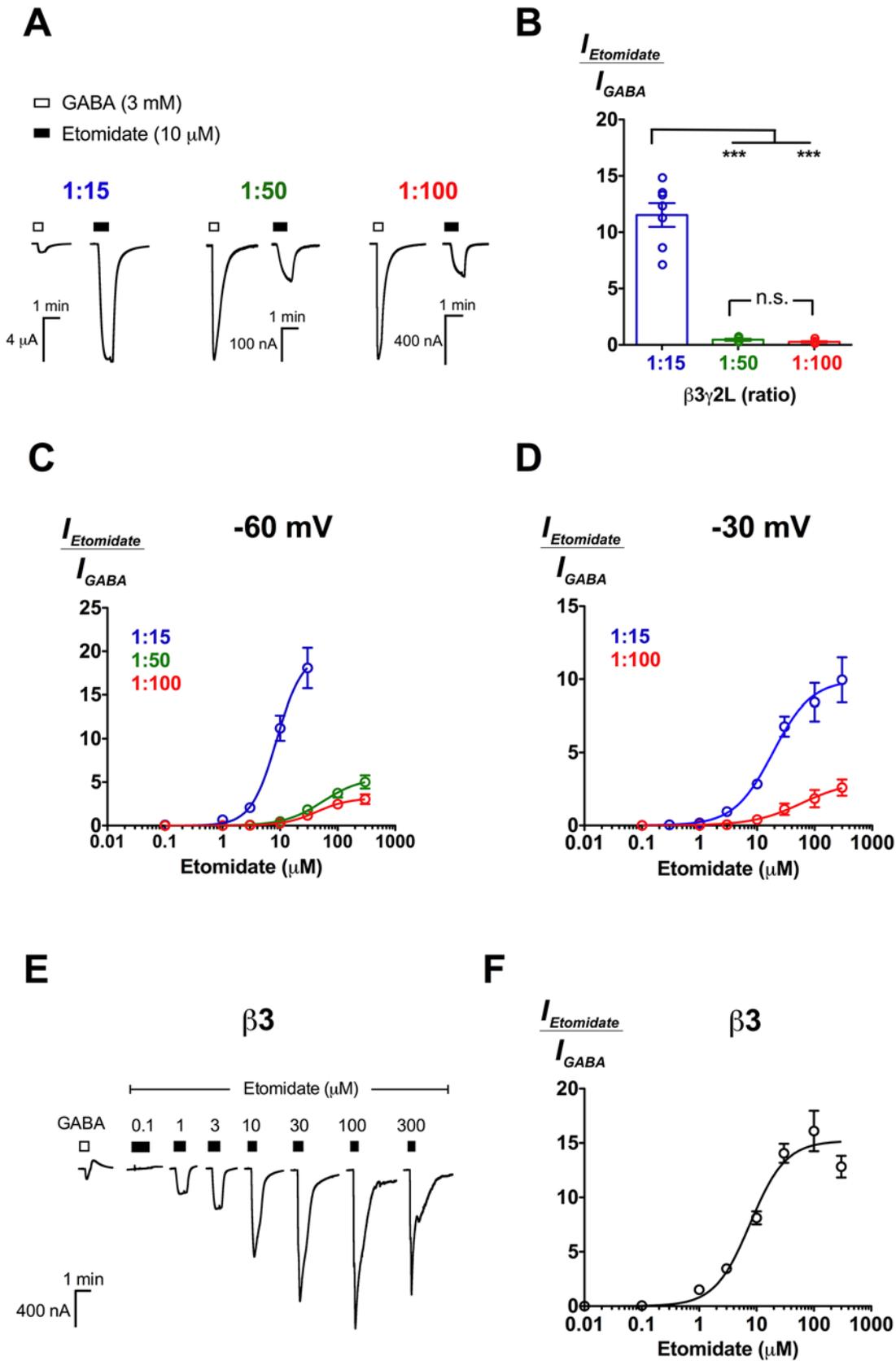
<sup>e</sup> 30  $\mu M$  (for 1:15) and 300  $\mu M$  (for 1:50 and 1:100) etomidate-elicited current normalised against 3 mM GABA response.

<sup>f</sup> 300  $\mu M$  etomidate-elicited current normalised against 3 mM GABA response.

<sup>g</sup> N.D. = not determined.

<sup>h</sup> 300  $\mu M$  propofol-elicited current normalised against 3 mM GABA response.

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**Fig 5. Etomidate activates both  $\beta 3\gamma 2L$  and  $\beta 3$  GABA<sub>A</sub>Rs.** (A) Representative traces of etomidate (10  $\mu M$ ) direct activation in comparison to 3 mM GABA at  $\beta 3\gamma 2L$  (1:15), (1:50) and (1:100) GABA<sub>A</sub>Rs. (B) Mean efficacy of 10  $\mu M$  etomidate activation at  $\beta 3\gamma 2L$  (1:15;  $n = 7$ ), (1:50;  $n = 6$ ) and (1:100;  $n = 7$ ) receptors. Data are normalised to the 3 mM GABA response. The mean efficacy of 10  $\mu M$  etomidate at various ratios was compared using Tukey's test, and the significance levels are indicated with n.s. (not significant) and \*\*\* ( $p \leq 0.001$ ). (C) Concentration-response curves of etomidate activation at  $\beta 3\gamma 2L$  receptors expressed at 1:15 ( $n = 5$ ), 1:50 ( $n = 6$ ) and 1:100 ( $n = 7$ ) ratios. Recording was conducted at -60 mV. Data are normalised to the 3 mM GABA response. (D) Concentration-response curves of etomidate activation at  $\beta 3\gamma 2L$  receptors expressed at 1:15 ( $n = 4$ ) and 1:100 ( $n = 5$ ) ratios. Recording was conducted at -30 mV. Data are normalised to the 3 mM GABA response. (E) Representative traces of  $\beta 3$  homomeric receptors responses to 0.1–300  $\mu M$  etomidate in comparison to 60 mM GABA response. (F) Etomidate concentration-response curve for  $\beta 3$  homomeric receptors ( $n = 5$ ). Data are normalised against 60 mM GABA responses. Data are presented as mean  $\pm$  SEM. Bars indicate durations of drug application. The holding current values are represented by the dotted lines.

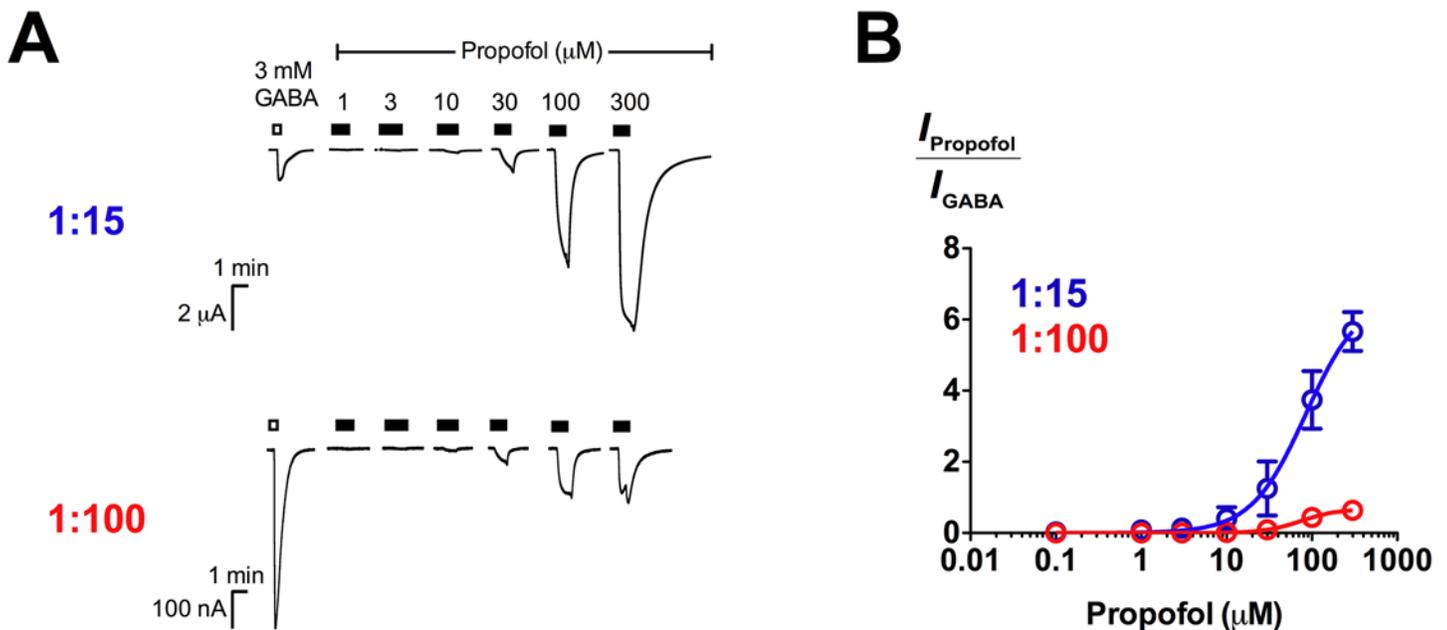
doi:10.1371/journal.pone.0141359.g005

but significantly higher potency at 1:15 ( $EC_{50} = 19 \mu M$ ) than 1:100 ( $EC_{50} = 60 \mu M$ ) ratio ( $p \leq 0.01$  for  $\log EC_{50}$  comparison; unpaired  $t$  test; Table 2).

We also investigated the action of propofol, a non-selective general anaesthetic which has been shown to bind with similar affinity to at least 4 binding sites at GABA<sub>A</sub>Rs found on  $\beta + \alpha -$ ,  $\alpha + \beta -$ ,  $\beta + \beta -$  and  $\gamma + \beta -$  subunit interfaces [42, 43]. We found that propofol directly activated  $\beta 3\gamma 2L$  receptors, in agreement with previous findings reported on  $\beta 2\gamma 2S$  receptors [26]. Like etomidate, propofol directly activated  $\beta 3\gamma 2L$  (1:15) and (1:100) receptors differentially (Fig 6A). At 300  $\mu M$ , propofol elicited current responses approximately 6-fold larger than maximal GABA currents at  $\beta 3\gamma 2L$  (1:15) receptors, significantly larger than at  $\beta 3\gamma 2L$  (1:100) receptors ( $E_{max} = 0.64 \pm 0.23$ ;  $p \leq 0.001$ ; unpaired  $t$  test; Fig 6B; Table 2). However, the potencies of propofol measured at 1:15 ( $EC_{50} = 85 \mu M$ ) and 1:100 ( $EC_{50} = 74 \mu M$ ) ratios were not different ( $p > 0.05$ ; unpaired  $t$  test; Table 2).

### Discussion

2'MeO6MF has been previously demonstrated to directly activate recombinant  $\alpha 2\beta 2/3\gamma 2L$  GABA<sub>A</sub>Rs expressed in *Xenopus* oocytes [30], but the functional significance of each subunit



**Fig 6. Propofol activates  $\beta 3\gamma 2L$  (1:15) and (1:100) receptors with different relative efficacies.** (A) Representative traces of propofol (1–300  $\mu M$ ) direct activation in comparison to 3 mM GABA at  $\beta 3\gamma 2L$  (1:15) and (1:100) GABA<sub>A</sub>Rs. (B) Concentration-response curves of propofol activation at  $\beta 3\gamma 2L$  receptors expressed at 1:15 ( $n = 4$ ) and 1:100 ( $n = 4$ ) ratios. Data are normalised to the 3 mM GABA response.

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remains unclear. In this study, we performed a systematic investigation of 2'MeO6MF activity at all receptor combinations expressed in *Xenopus* oocytes using  $\alpha 2$ ,  $\beta 3$  and  $\gamma 2L$  subunits and found that 2'MeO6MF exhibits distinct pharmacology at  $\beta 3$ ,  $\beta 3\gamma 2L$  and  $\alpha 2\beta 3\gamma 2L$  GABA<sub>A</sub>Rs. It acts as an inverse agonist at  $\beta 3$  homomers by inhibiting the constitutive activity of the receptors, and activates  $\beta 3\gamma 2L$  and  $\alpha 2\beta 3\gamma 2L$  GABA<sub>A</sub>Rs with different relative efficacies. The lack of activity of 2'MeO6MF at  $\alpha 2\beta 3$  (20:1) binary receptors, despite the presence of the  $\beta 3$  subunit, favours an interfacial 2'MeO6MF binding site(s) containing the  $\beta 3$  subunit ( $\beta 3$ - $\beta 3$ ,  $\beta 3$ - $\gamma 2L$  or  $\gamma 2L$ - $\beta 3$ ) over an intra- $\beta 3$ -subunit binding site.

The robust formation of the non- $\alpha$ -containing  $\beta 3\gamma 2L$  GABA<sub>A</sub>Rs raises a critical question of where GABA binds to activate this receptor as it lacks the  $\beta + \alpha$ - interfaces. Despite the absence of any known GABA binding sites in  $\beta 3\gamma 2L$  receptors, GABA elicited robust responses within a similar concentration range that activates  $\alpha \beta$  and  $\alpha \beta \gamma$  receptors. Thus, we infer that GABA binding site(s) which are previously unknown may exist. A GABA binding site at the  $\beta$ - $\beta$  interface is possible, but the poor GABA activity at  $\beta 3$  homomeric receptors even at saturating concentrations suggests that other subunit interfaces, possibly between the  $\beta$  and  $\gamma$  subunits are more likely to contribute to the GABA sensitivity of  $\beta 3\gamma 2L$  receptors.

There is a wealth of evidence supporting two  $\beta + \alpha$ - interfacial binding sites for etomidate in the transmembrane domain [11–13]. Our data show that etomidate activates  $\beta 3$  homomeric and  $\beta 3\gamma 2L$  binary receptors efficaciously, but is insensitive at  $\gamma 2L$  homomeric receptors. These findings indicate that the  $\beta$ , but not the  $\alpha$  subunit is essential for etomidate sensitivity. This is supported by findings from a photoaffinity labelling study which suggested that etomidate can also bind to a homologous  $\beta + \beta$ - interfacial site with less selectivity [43]. Besides that, mutations found on the  $\beta$  subunit such as N265M [33] and M286W [44] have been shown to completely eliminate etomidate sensitivity, while mutations found on the  $\alpha$  subunit tend to have less profound effects [45]. Taken together, we infer from these findings that the  $\beta$  subunit, which bears the principal (+) components of the etomidate binding sites, dominates ligand binding interactions, whereas the identity of the complementary (–) subunit ( $\alpha$ ,  $\beta$  or  $\gamma$ ) is well tolerated.

We also demonstrate that  $\beta 3\gamma 2L$  receptors expressed at different injection ratios exhibit distinct levels of constitutive activity, relative efficacies of 2'MeO6MF, etomidate and propofol activation as well as different GABA potencies. These findings strongly suggest that  $\beta 3\gamma 2L$  receptors with different subunit stoichiometries are being formed. The influence of subunit cDNA or mRNA ratios on receptor stoichiometry expressed in heterologous systems is well established in the case of  $\alpha 4\beta 2$  nicotinic acetylcholine receptors, in which  $\alpha 4$ -biased and  $\beta 2$ -biased ratios are routinely used to selectively express the pharmacologically dissimilar 3 ( $\alpha 4$ ):2( $\beta 2$ ) and 2( $\alpha 4$ ):3( $\beta 2$ ) stoichiometries respectively [46, 47]. It is not clear from our experiments which stoichiometries of  $\beta 3\gamma 2L$  receptors exist. However, as the binary  $\alpha \beta$  GABA<sub>A</sub>Rs have been shown to assemble into two functional stoichiometries of 2 $\alpha$ :3 $\beta$  and 3 $\alpha$ :2 $\beta$  [6, 21, 22], by analogy, we propose that  $\beta 3\gamma 2L$  GABA<sub>A</sub>Rs also exist in two different stoichiometries of 3 $\beta$ :2 $\gamma$  and 2 $\beta$ :3 $\gamma$ . It is likely that when the  $\gamma 2L$  subunit mRNA was injected in greater abundance relative to the  $\beta 3$  subunit (e.g., 1:50 and 1:100 ratios), the 2 $\beta$ :3 $\gamma$  stoichiometry is predominantly expressed. In contrast, when the injection ratio was reduced (e.g., 1:15), the 3 $\beta$ :2 $\gamma$  stoichiometry may be favourably expressed. The definitive mechanisms underlying the distinct pharmacological profiles of  $\beta 3\gamma 2L$  stoichiometric forms are beyond the scope of this investigation, but differences in the number of functional binding interfaces and intrinsic activation properties may have a role. Further experiments are required to understand how these factors result in the complex pharmacology observed for  $\beta 3\gamma 2L$  receptors.

We and others have shown that 2'MeO6MF, etomidate, pentobarbital and dopamine appear to be more efficacious agonists than GABA at  $\beta 3\gamma 2$  receptors [27, 29]. In contrast, these ligands

directly activate  $\alpha\beta\gamma$  receptors with lower efficacies relative to the maximal GABA responses [29, 44, 48]. Similar observations have been reported at  $\alpha\beta\delta$  receptors, where GABA acts as a partial agonist with low intrinsic efficacy, and modulators such as neurosteroids [49], etomidate [50], propofol [51] and pentobarbital [48] are able to enhance maximal GABA currents significantly larger than they do at  $\alpha\beta\gamma$  receptors, where GABA acts as a full agonist. It is possible that GABA also acts as a weak partial agonist at  $\beta\gamma$  receptors. However, this inference is not conclusive as the intrinsic efficacies of GABA and other ligands at  $\beta\gamma$  receptors are not well established and were not systematically explored in our study. Further studies are required to address these issues, and to clarify whether the apparent larger relative efficacies of various ligands at  $\beta\gamma$  receptors are due to subtype-dependent functional differences or the low GABA efficacy at these channels.

The expression of  $\beta\gamma$  receptors has been reported previously [23–29]. In these studies, the overall pharmacological profiles of  $\beta\gamma$  receptors are very similar to that of the  $\alpha\beta\gamma$  receptors. This is concerning as pharmacological tools such as diazepam, which is routinely used as an indicator of the purity of the  $\alpha\beta\gamma$  receptors heterologously expressed would not be able to distinguish  $\beta\gamma$  from  $\alpha\beta\gamma$  receptors due to its comparable actions at both receptor subtypes [23, 25]. The GABA<sub>A</sub>R competitive antagonist bicuculline has recently been suggested to have  $\beta 3\gamma 2$ -selective pharmacology as it exhibits inhibitory action at  $\alpha 1\beta 3\gamma 2$  but activates  $\beta 3\gamma 2$  receptors [29]. However, as bicuculline has been shown to also activate  $\beta 3$  homomers [39], it is not known if the effect observed at  $\beta 3\gamma 2$  receptors was mediated by a background population of  $\beta 3$  homomers. The great diversity in GABA<sub>A</sub>R subtypes dictates the need for subtype-specific ligands to understand the function of these receptors. As such, the search for  $\beta\gamma$ -selective ligands is pivotal to accurately determine the pharmacology of these receptors *in vitro*.

There is emerging evidence to suggest that native GABA<sub>A</sub>R subtypes are far more diverse than earlier studies have indicated, as exemplified by the *in vivo* expression of  $\alpha\beta$  receptors which were previously thought to only form in heterologous systems [52–56]. In single channel recording experiments conducted in dissociated rat hippocampal neurons, it has been shown that the activity of 2'MeO6MF, like diazepam, is significantly attenuated by the  $\gamma 2(381-403)$  MA peptide which disrupts protein-receptor interactions involving only the  $\gamma 2$  subunit [30, 57]. This finding suggests that  $\gamma 2$ -containing GABA<sub>A</sub>Rs are the molecular target of 2'MeO6MF, and the subunit composition of these receptors is most likely to make up of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits since they are the most common native isoforms. It is currently not known if  $\beta\gamma$  receptors exist in neurons and thus contribute partially to the flavonoid activation observed. Again,  $\beta\gamma$ -selective ligands will be necessary to explore this possibility.

## Conclusions

Overall, our findings have important implications in the study of GABA<sub>A</sub>Rs. We have shown that  $\beta 3\gamma 2L$  receptors assemble with different subunit stoichiometries in *Xenopus* oocytes, which can be differentiated by GABA, 2'MeO6MF, etomidate and propofol. These receptors lack the  $\alpha$  subunit which contributes the complementary (–) components of the GABA and etomidate binding sites, but are still activated by GABA and etomidate, suggesting that the structural requirements for these sites need to be redefined. While the *in vivo* existence of  $\beta\gamma$  receptors remains elusive, it can serve as a tool to study the contribution of different subunits or subunit interfaces in the pharmacology of a chemical of interest.

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## Author Contributions

Conceived and designed the experiments: HC NA MC. Performed the experiments: HC. Analyzed the data: HC NA MC. Contributed reagents/materials/analysis tools: JRH RV. Wrote the paper: HC NA MC.

## References

- Olsen RW, Sieghart W. International Union of Pharmacology. LXX. Subtypes of  $\gamma$ -aminobutyric acid<sub>A</sub> receptors: classification on the basis of subunit composition, pharmacology, and function. Update. *Pharmacological Reviews*. 2008; 60(3):243–60. doi: [10.1124/pr.108.00505](https://doi.org/10.1124/pr.108.00505) PMID: [18790874](https://pubmed.ncbi.nlm.nih.gov/18790874/)
- Farrant M, Nusser Z. Variations on an inhibitory theme: phasic and tonic activation of GABA<sub>A</sub> receptors. *Nature Reviews Neuroscience*. 2005; 6(3):215–29. PMID: [15738957](https://pubmed.ncbi.nlm.nih.gov/15738957/)
- Jacob TC, Moss SJ, Jurd R. GABA<sub>A</sub> receptor trafficking and its role in the dynamic modulation of neuronal inhibition. *Nature Reviews Neuroscience*. 2008; 9(5):331–43. doi: [10.1038/nrn2370](https://doi.org/10.1038/nrn2370) PMID: [18382465](https://pubmed.ncbi.nlm.nih.gov/18382465/)
- Luscher B, Fuchs T, Kilpatrick Casey L. GABA<sub>A</sub> receptor trafficking-mediated plasticity of inhibitory synapses. *Neuron*. 2011; 70(3):385–409. doi: [10.1016/j.neuron.2011.03.024](https://doi.org/10.1016/j.neuron.2011.03.024) PMID: [21555068](https://pubmed.ncbi.nlm.nih.gov/21555068/)
- Tretter VE, Mukherjee J, Maric HM, Schindelin H, Sieghart W, Moss SJ. Gephyrin, the enigmatic organizer at GABAergic synapses. *Frontiers in Cellular Neuroscience*. 2012; 6(23).
- Baumann SW, Baur R, Sigel E. Subunit arrangement of  $\gamma$ -aminobutyric acid type A receptors. *Journal of Biological Chemistry*. 2001; 276(39):36275–80. PMID: [11466317](https://pubmed.ncbi.nlm.nih.gov/11466317/)
- Baumann SW, Baur R, Sigel E. Forced subunit assembly in  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub> receptors: insight into the absolute arrangement. *Journal of Biological Chemistry*. 2002; 277(48):46020–5. PMID: [12324466](https://pubmed.ncbi.nlm.nih.gov/12324466/)
- Baur R, Minier F, Sigel E. A GABA<sub>A</sub> receptor of defined subunit composition and positioning: concatenation of five subunits. *FEBS Letters*. 2006; 580(6):1616–20. PMID: [16494876](https://pubmed.ncbi.nlm.nih.gov/16494876/)
- Smith GB, Olsen RW. Functional domains of GABA<sub>A</sub> receptors. *Trends in Pharmacological Sciences*. 1995; 16(5):162–8. PMID: [7624971](https://pubmed.ncbi.nlm.nih.gov/7624971/)
- Sigel E, Buhr A. The benzodiazepine binding site of GABA<sub>A</sub> receptors. *Trends in Pharmacological Sciences*. 1997; 18(11):425–9. PMID: [9426470](https://pubmed.ncbi.nlm.nih.gov/9426470/)
- Sieghart W. Chapter Three—Allosteric modulation of GABA<sub>A</sub> receptors via multiple drug-binding sites. In: Uwe R, editor. *Advances in Pharmacology*. Volume 72: Academic Press; 2015. p. 53–96.
- Li G-D, Chiara DC, Sawyer GW, Husain SS, Olsen RW, Cohen JB. Identification of a GABA<sub>A</sub> receptor anesthetic binding site at subunit interfaces by photolabeling with an etomidate analog. *The Journal of Neuroscience*. 2006; 26(45):11599–605. PMID: [17093081](https://pubmed.ncbi.nlm.nih.gov/17093081/)
- Chiara DC, Dostalova Z, Jayakar SS, Zhou X, Miller KW, Cohen JB. Mapping general anesthetic binding site(s) in human  $\alpha 1\beta 3\gamma$   $\gamma$ -aminobutyric acid type A receptors with [<sup>3</sup>H]TDBzl-etomidate, a photoreactive etomidate analogue. *Biochemistry*. 2012; 51(4):836–47. doi: [10.1021/bi201772m](https://doi.org/10.1021/bi201772m) PMID: [22243422](https://pubmed.ncbi.nlm.nih.gov/22243422/)
- Ramerstorfer J, Furtmüller R, Sarto-Jackson I, Varagic Z, Sieghart W, Ernst M. The GABA<sub>A</sub> receptor  $\alpha +\beta$ - interface: a novel target for subtype selective drugs. *The Journal of Neuroscience*. 2011; 31(3):870–7. doi: [10.1523/JNEUROSCI.5012-10.2011](https://doi.org/10.1523/JNEUROSCI.5012-10.2011) PMID: [21248110](https://pubmed.ncbi.nlm.nih.gov/21248110/)
- Sieghart W, Ramerstorfer J, Sarto-Jackson I, Varagic Z, Ernst M. A novel GABA<sub>A</sub> receptor pharmacology: drugs interacting with the  $\alpha +\beta$ - interface. *British Journal of Pharmacology*. 2012; 166(2):476–85. doi: [10.1111/j.1476-5381.2011.01779.x](https://doi.org/10.1111/j.1476-5381.2011.01779.x) PMID: [22074382](https://pubmed.ncbi.nlm.nih.gov/22074382/)
- Varagic Z, Ramerstorfer J, Huang S, Rallapalli S, Sarto-Jackson I, Cook J, et al. Subtype selectivity of  $\alpha +\beta$ - site ligands of GABA<sub>A</sub> receptors: identification of the first highly specific positive modulators at  $\alpha 6\beta 2/3\gamma 2$  receptors. *British Journal of Pharmacology*. 2013; 169(2):384–99. doi: [10.1111/bph.12153](https://doi.org/10.1111/bph.12153) PMID: [23472935](https://pubmed.ncbi.nlm.nih.gov/23472935/)
- Varagic Z, Wimmer L, Schnürch M, Mihovilovic MD, Huang S, Rallapalli S, et al. Identification of novel positive allosteric modulators and null modulators at the GABA<sub>A</sub> receptor  $\alpha +\beta$ - interface. *British Journal of Pharmacology*. 2013; 169(2):371–83. doi: [10.1111/bph.12151](https://doi.org/10.1111/bph.12151) PMID: [23472852](https://pubmed.ncbi.nlm.nih.gov/23472852/)
- Jayakar SS, Zhou X, Savechenkov PY, Chiara DC, Desai R, Bruzik KS, et al. Positive and negative allosteric modulation of an  $\alpha 1\beta 3\gamma 2$   $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>) receptor by binding to a site in the transmembrane domain at the  $\gamma^+$ - $\beta$  interface. *Journal of Biological Chemistry*. 2015.
- Boileau AJ, Baur R, Sharkey LM, Sigel E, Czajkowski C. The relative amount of cRNA coding for  $\gamma 2$  subunits affects stimulation by benzodiazepines in GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes. *Neuropharmacology*. 2002; 43(4):695–700. PMID: [12367615](https://pubmed.ncbi.nlm.nih.gov/12367615/)

20. Baburin I, Khom S, Timin E, Hohaus A, Sieghart W, Hering S. Estimating the efficiency of benzodiazepines on GABA<sub>A</sub> receptors comprising  $\gamma 1$  or  $\gamma 2$  subunits. *British Journal of Pharmacology*. 2008; 155(3):424–33. doi: [10.1038/bjp.2008.271](https://doi.org/10.1038/bjp.2008.271) PMID: [18604239](https://pubmed.ncbi.nlm.nih.gov/18604239/)
21. Im WB, Pregenzer JF, Binder JA, Dillon GH, Alberts GL. Chloride channel expression with the tandem construct of  $\alpha 6$ - $\beta 2$  GABA<sub>A</sub> receptor subunit requires a monomeric subunit of  $\alpha 6$  or  $\gamma 2$ . *Journal of Biological Chemistry*. 1995; 270(44):26063–6. PMID: [7592805](https://pubmed.ncbi.nlm.nih.gov/7592805/)
22. Boileau AJ, Pearce RA, Czajkowski C. Tandem subunits effectively constrain GABA<sub>A</sub> receptor stoichiometry and recapitulate receptor kinetics but are insensitive to GABA<sub>A</sub> receptor-associated protein. *The Journal of Neuroscience*. 2005; 25(49):11219–30. PMID: [16339017](https://pubmed.ncbi.nlm.nih.gov/16339017/)
23. Sigel E, Baur R, Trube G, Möhler H, Malherbe P. The effect of subunit composition of rat brain GABA<sub>A</sub> receptors on channel function. *Neuron*. 1990; 5(5):703–11. PMID: [1699569](https://pubmed.ncbi.nlm.nih.gov/1699569/)
24. Knoflach F, Backus KH, Giller T, Malherbe P, Pflimlin P, Möhler H, et al. Pharmacological and electrophysiological properties of recombinant GABA<sub>A</sub> receptors comprising the  $\alpha 3$ ,  $\beta 1$  and  $\gamma 2$  subunits. *European Journal of Neuroscience*. 1992; 4(1):1–9. PMID: [12106436](https://pubmed.ncbi.nlm.nih.gov/12106436/)
25. Whittemore ER, Yang W, Drewe JA, Woodward RM. Pharmacology of the human gamma-aminobutyric acid<sub>A</sub> receptor  $\alpha 4$  subunit expressed in *Xenopus laevis* oocytes. *Molecular Pharmacology*. 1996; 50(5):1364–75. PMID: [8913369](https://pubmed.ncbi.nlm.nih.gov/8913369/)
26. Sanna E, Motzo C, Usala M, Serra M, Dazzi L, Maciocco E, et al. Characterization of the electrophysiological and pharmacological effects of 4-iodo-2,6-diisopropylphenol, a propofol analogue devoid of sedative-anaesthetic properties. *British Journal of Pharmacology*. 1999; 126(6):1444–54. PMID: [10217539](https://pubmed.ncbi.nlm.nih.gov/10217539/)
27. Taylor PM, Thomas P, Gorrie GH, Connolly CN, Smart TG, Moss SJ. Identification of amino acid residues within GABA<sub>A</sub> receptor  $\beta$  subunits that mediate both homomeric and heteromeric receptor expression. *The Journal of Neuroscience*. 1999; 19(15):6360–71. PMID: [10414965](https://pubmed.ncbi.nlm.nih.gov/10414965/)
28. Miko A, Werby E, Sun H, Healey J, Zhang L. A TM2 residue in the  $\beta 1$  subunit determines spontaneous opening of homomeric and heteromeric  $\gamma$ -aminobutyric acid-gated ion channels. *Journal of Biological Chemistry*. 2004; 279(22):22833–40. PMID: [15014066](https://pubmed.ncbi.nlm.nih.gov/15014066/)
29. Hoerbel P, Lindsley TA, Fleck MW. Dopamine directly modulates GABA<sub>A</sub> receptors. *The Journal of Neuroscience*. 2015; 35(8):3525–36. doi: [10.1523/JNEUROSCI.4390-14.2015](https://doi.org/10.1523/JNEUROSCI.4390-14.2015) PMID: [25716851](https://pubmed.ncbi.nlm.nih.gov/25716851/)
30. Karim N, Curmi J, Gavande N, Johnston GA, Hanrahan JR, Tierney ML, et al. 2'-Methoxy-6-methylflavone: a novel anxiolytic and sedative with subtype selective activating and modulating actions at GABA<sub>A</sub> receptors. *British Journal of Pharmacology*. 2012; 165(4):880–96. doi: [10.1111/j.1476-5381.2011.01604.x](https://doi.org/10.1111/j.1476-5381.2011.01604.x) PMID: [21797842](https://pubmed.ncbi.nlm.nih.gov/21797842/)
31. Belelli D, Lambert JJ, Peters JA, Wafford K, Whiting PJ. The interaction of the general anesthetic etomidate with the  $\gamma$ -aminobutyric acid type A receptor is influenced by a single amino acid. *Proceedings of the National Academy of Sciences of the United States of America*. 1997; 94(20):11031–6. PMID: [9380754](https://pubmed.ncbi.nlm.nih.gov/9380754/)
32. Stewart DS, Pierce DW, Hotta M, Stern AT, Forman SA. Mutations at beta N265 in  $\gamma$ -aminobutyric acid type A receptors alter both binding affinity and efficacy of potent anesthetics. *PloS one*. 2014; 9(10):e111470. doi: [10.1371/journal.pone.0111470](https://doi.org/10.1371/journal.pone.0111470) PMID: [25347186](https://pubmed.ncbi.nlm.nih.gov/25347186/)
33. Desai R, Ruesch D, Forman Stuart A.  $\gamma$ -Amino butyric acid type A receptor mutations at  $\beta 2N265$  alter etomidate efficacy while preserving basal and agonist-dependent activity. *Anesthesiology*. 2009; 111(4):774–84. doi: [10.1097/ALN.0b013e3181b55fae](https://doi.org/10.1097/ALN.0b013e3181b55fae) PMID: [19741491](https://pubmed.ncbi.nlm.nih.gov/19741491/)
34. Janssen PA, Niemegeers CJ, Schellekens KH, Lenaerts FM. Etomidate, R-(+)-ethyl-1-( $\alpha$ -methyl-benzyl)imidazole-5-carboxylate (R 16659), a potent, short-acting and relatively atoxic intravenous hypnotic agent in rats. *Arzneimittel-Forschung*. 1971; 21(8):1234–43. PMID: [5110026](https://pubmed.ncbi.nlm.nih.gov/5110026/)
35. Janssen CGM, Thijssen JBA, Verluyten WLM, Heykants JJP. Synthesis of (R)-(+)-<sup>3</sup>H-etomidate. *Journal of Labelled Compounds and Radiopharmaceuticals*. 1987; 24(8):909–18.
36. Walters RJ, Hadley SH, Morris KD, Amin J. Benzodiazepines act on GABA<sub>A</sub> receptors via two distinct and separable mechanisms. *Nat Neurosci*. 2000; 3(12):1274–81. PMID: [11100148](https://pubmed.ncbi.nlm.nih.gov/11100148/)
37. Masneuf S, Buetler J, Koester C, Crestani F. Role of  $\alpha 1$ - and  $\alpha 2$ -GABA<sub>A</sub> receptors in mediating the respiratory changes associated with benzodiazepine sedation. *British Journal of Pharmacology*. 2012; 166(1):339–48. doi: [10.1111/j.1476-5381.2011.01763.x](https://doi.org/10.1111/j.1476-5381.2011.01763.x) PMID: [22044283](https://pubmed.ncbi.nlm.nih.gov/22044283/)
38. Joksimović S, Varagic Z, Kovačević J, Van Linn M, Milić M, Rallapalli S, et al. Insights into functional pharmacology of  $\alpha 1$  GABA<sub>A</sub> receptors: how much does partial activation at the benzodiazepine site matter? *Psychopharmacology*. 2013; 230(1):113–23. doi: [10.1007/s00213-013-3143-4](https://doi.org/10.1007/s00213-013-3143-4) PMID: [23685860](https://pubmed.ncbi.nlm.nih.gov/23685860/)
39. Wooltorton JR, Moss SJ, Smart TG. Pharmacological and physiological characterization of murine homomeric  $\beta 3$  GABA<sub>A</sub> receptors. *European Journal of Neuroscience*. 1997; 9(11):2225–35. PMID: [9464918](https://pubmed.ncbi.nlm.nih.gov/9464918/)

40. Saras A, Gisselmann G, Vogt-Eisele AK, Erkkamp KS, Kletke O, Pusch H, et al. Histamine action on vertebrate GABA<sub>A</sub> receptors: direct channel gating and potentiation of GABA responses. *Journal of Biological Chemistry*. 2008; 283(16):10470–5. doi: [10.1074/jbc.M709993200](https://doi.org/10.1074/jbc.M709993200) PMID: [18281286](https://pubmed.ncbi.nlm.nih.gov/18281286/)
41. O'Toole KK, Jenkins A. The apparent voltage dependence of GABA<sub>A</sub> receptor activation and modulation is inversely related to channel open probability. *Molecular Pharmacology*. 2012; 81(2):189–97. doi: [10.1124/mol.111.074476](https://doi.org/10.1124/mol.111.074476) PMID: [22042665](https://pubmed.ncbi.nlm.nih.gov/22042665/)
42. Chiara DC, Jayakar SS, Zhou X, Zhang X, Savechenkov PY, Bruzik KS, et al. Specificity of intersubunit general anesthetic-binding sites in the transmembrane domain of the human  $\alpha 1\beta 3\gamma 2$   $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>) receptor. *Journal of Biological Chemistry*. 2013; 288(27):19343–57. doi: [10.1074/jbc.M113.479725](https://doi.org/10.1074/jbc.M113.479725) PMID: [23677991](https://pubmed.ncbi.nlm.nih.gov/23677991/)
43. Jayakar SS, Zhou X, Chiara DC, Dostalova Z, Savechenkov PY, Bruzik KS, et al. Multiple propofol-binding sites in a  $\gamma$ -aminobutyric acid type A receptor (GABA<sub>A</sub>R) identified using a photoreactive propofol analog. *Journal of Biological Chemistry*. 2014; 289(40):27456–68. doi: [10.1074/jbc.M114.581728](https://doi.org/10.1074/jbc.M114.581728) PMID: [25086038](https://pubmed.ncbi.nlm.nih.gov/25086038/)
44. Stewart D, Desai R, Cheng Q, Liu A, Forman SA. Tryptophan mutations at azi-etomidate photo-incorporation sites on  $\alpha 1$  or  $\beta 2$  subunits enhance GABA<sub>A</sub> receptor gating and reduce etomidate modulation. *Molecular Pharmacology*. 2008; 74(6):1687–95. doi: [10.1124/mol.108.050500](https://doi.org/10.1124/mol.108.050500) PMID: [18805938](https://pubmed.ncbi.nlm.nih.gov/18805938/)
45. Stewart DS, Hotta M, Li GD, Desai R, Chiara DC, Olsen RW, et al. Cysteine substitutions define etomidate binding and gating linkages in the  $\alpha$ -M1 domain of  $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>) receptors. *The Journal of Biological Chemistry*. 2013; 288(42):30373–86. doi: [10.1074/jbc.M113.494583](https://doi.org/10.1074/jbc.M113.494583) PMID: [24009076](https://pubmed.ncbi.nlm.nih.gov/24009076/)
46. Harpsøe K, Ahring PK, Christensen JK, Jensen ML, Peters D, Balle T. Unraveling the high- and low-sensitivity agonist responses of nicotinic acetylcholine receptors. *The Journal of Neuroscience*. 2011; 31(30):10759–66. doi: [10.1523/JNEUROSCI.1509-11.2011](https://doi.org/10.1523/JNEUROSCI.1509-11.2011) PMID: [21795528](https://pubmed.ncbi.nlm.nih.gov/21795528/)
47. Mazzaferro S, Gasparri F, New K, Alcaïno C, Faundez M, Iturriaga Vasquez P, et al. Non-equivalent ligand selectivity of agonist sites in  $(\alpha 4\beta 2)_2\alpha 4$  nicotinic acetylcholine receptors: a key determinant of agonist efficacy. *Journal of Biological Chemistry*. 2014; 289(31):21795–806. doi: [10.1074/jbc.M114.555136](https://doi.org/10.1074/jbc.M114.555136) PMID: [24936069](https://pubmed.ncbi.nlm.nih.gov/24936069/)
48. Feng H-J, Bianchi MT, Macdonald RL. Pentobarbital differentially modulates  $\alpha 1\beta 3\delta$  and  $\alpha 1\beta 3\gamma 2L$  GABA<sub>A</sub> receptor currents. *Molecular Pharmacology*. 2004; 66(4):988–1003. PMID: [15247320](https://pubmed.ncbi.nlm.nih.gov/15247320/)
49. Bianchi MT, Macdonald RL. Neurosteroids shift partial agonist activation of GABA<sub>A</sub> receptor channels from low- to high-efficacy gating patterns. *The Journal of Neuroscience*. 2003; 23(34):10934–43. PMID: [14645489](https://pubmed.ncbi.nlm.nih.gov/14645489/)
50. Feng HJ, Jounaidi Y, Haburcak M, Yang X, Forman SA. Etomidate produces similar allosteric modulation in  $\alpha 1\beta 3\delta$  and  $\alpha 1\beta 3\gamma 2L$  GABA<sub>A</sub> receptors. *British Journal of Pharmacology*. 2014; 171(3):789–98. doi: [10.1111/bph.12507](https://doi.org/10.1111/bph.12507) PMID: [24199598](https://pubmed.ncbi.nlm.nih.gov/24199598/)
51. Feng H-J, Macdonald RL. Multiple actions of propofol on  $\alpha\beta\gamma$  and  $\alpha\beta\delta$  GABA<sub>A</sub> receptors. *Molecular Pharmacology*. 2004; 66(6):1517–24. PMID: [15331770](https://pubmed.ncbi.nlm.nih.gov/15331770/)
52. Günther U, Benson J, Benke D, Fritschy JM, Reyes G, Knoflach F, et al. Benzodiazepine-insensitive mice generated by targeted disruption of the  $\gamma 2$  subunit gene of  $\gamma$ -aminobutyric acid type A receptors. *Proceedings of the National Academy of Sciences*. 1995; 92(17):7749–53.
53. Bencsits E, Ebert V, Tretter V, Sieghart W. A significant part of native  $\gamma$ -aminobutyric acid<sub>A</sub> receptors containing  $\alpha 4$  subunits do not contain  $\gamma$  or  $\delta$  subunits. *Journal of Biological Chemistry*. 1999; 274(28):19613–6. PMID: [10391897](https://pubmed.ncbi.nlm.nih.gov/10391897/)
54. Brickley SG, Cull-Candy SG, Farrant M. Single-channel properties of synaptic and extrasynaptic GABA<sub>A</sub> receptors suggest differential targeting of receptor subtypes. *The Journal of Neuroscience*. 1999; 19(8):2960–73. PMID: [10191314](https://pubmed.ncbi.nlm.nih.gov/10191314/)
55. Yeung JYT, Canning KJ, Zhu G, Pennefather P, MacDonald JF, Orser BA. Tonically activated GABA<sub>A</sub> receptors in hippocampal neurons are high-affinity, low-conductance sensors for extracellular GABA. *Molecular Pharmacology*. 2003; 63(1):2–8. PMID: [12488530](https://pubmed.ncbi.nlm.nih.gov/12488530/)
56. Mortensen M, Smart TG. Extrasynaptic  $\alpha\beta$  subunit GABA<sub>A</sub> receptors on rat hippocampal pyramidal neurons. *The Journal of Physiology*. 2006; 577(3):841–56.
57. Everitt AB, Seymour VAL, Curmi J, Laver DR, Gage PW, Tierney ML. Protein interactions involving the  $\gamma 2$  large cytoplasmic loop of GABA<sub>A</sub> receptors modulate conductance. *The FASEB Journal*. 2009; 23(12):4361–9. doi: [10.1096/fj.09-137042](https://doi.org/10.1096/fj.09-137042) PMID: [19703932](https://pubmed.ncbi.nlm.nih.gov/19703932/)