

## RESEARCH ARTICLE

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# Pharmacological characterisation of murine $\alpha 4\beta 1\delta$ GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes

Inge S Villumsen<sup>1,2\*</sup>, Petrine Wellendorph<sup>2</sup> and Trevor G Smart<sup>1</sup>**Abstract**

**Background:** GABA<sub>A</sub> receptor subunit composition has a profound effect on the receptor's physiological and pharmacological properties. The receptor  $\beta$  subunit is widely recognised for its importance in receptor assembly, trafficking and post-translational modifications, but its influence on extrasynaptic GABA<sub>A</sub> receptor function is less well understood. Here, we examine the pharmacological properties of a potentially native extrasynaptic GABA<sub>A</sub> receptor that incorporates the  $\beta 1$  subunit, specifically composed of  $\alpha 4\beta 1\delta$  and  $\alpha 4\beta 1$  subunits.

**Results:** GABA activated concentration-dependent responses at  $\alpha 4\beta 1\delta$  and  $\alpha 4\beta 1$  receptors with EC<sub>50</sub> values in the nanomolar to micromolar range, respectively. The divalent cations Zn<sup>2+</sup> and Cu<sup>2+</sup>, and the  $\beta 1$ -selective inhibitor salicylidine salicylhydrazide (SCS), inhibited GABA-activated currents at  $\alpha 4\beta 1\delta$  receptors. Surprisingly the  $\alpha 4\beta 1$  receptor demonstrated biphasic sensitivity to Zn<sup>2+</sup> inhibition that may reflect variable subunit stoichiometries with differing sensitivity to Zn<sup>2+</sup>. The neurosteroid tetrahydro-deoxycorticosterone (THDOC) significantly increased GABA-initiated responses in concentrations above 30 nM for  $\alpha 4\beta 1\delta$  receptors.

**Conclusions:** With this study we report the first pharmacological characterisation of various GABA<sub>A</sub> receptor ligands acting at murine  $\alpha 4\beta 1\delta$  GABA<sub>A</sub> receptors, thereby improving our understanding of the molecular pharmacology of this receptor isoform. This study highlights some notable differences in the pharmacology of murine and human  $\alpha 4\beta 1\delta$  receptors. We consider the likelihood that the  $\alpha 4\beta 1\delta$  receptor may play a role as an extrasynaptic GABA<sub>A</sub> receptor in the nervous system.

**Keywords:**  $\gamma$ -aminobutyric acid, GABA<sub>A</sub> receptors,  $\alpha 4\beta 1\delta$  subtype, Extrasynaptic receptors,  $\beta 1$  subunit

**Background**

GABA<sub>A</sub> receptors are pentameric ligand-gated ion channels that mediate phasic and tonic inhibition in the central nervous system [1]. When activated by GABA the integral ion channel opens, rendering the receptor permeable to chloride ions. The co-assembly of combinations of 19 different subunits ( $\alpha 1-6$ ,  $\beta 1-3$ ,  $\gamma 1-3$ ,  $\delta$ ,  $\epsilon$ ,  $\theta$ ,  $\pi$  and  $\rho 1-3$ ) into homo- or hetero-pentameric receptors results in multiple different GABA<sub>A</sub> receptor isoforms with different function, pharmacology and neuronal location [2-4]. The stoichiometry of the majority of these

receptors is considered to be  $2\alpha:2\beta:1\gamma/\delta$ , predominantly arranged as  $\beta-\alpha-\beta-\alpha-\gamma/\delta$  in an anticlockwise manner when viewed from the extracellular space [5-7]. However, the arrangement for  $\delta$  subunit-containing receptors seems to be dependent on the experimental conditions [6,8-12].

Receptors incorporating  $\alpha 1$ ,  $\alpha 2$  or  $\alpha 3$  subunits most commonly assemble with  $\beta$  and  $\gamma$  subunits, and are generally located at the synapse where they mediate phasic inhibition; whereas  $\alpha 4$  and  $\alpha 6$ -containing receptors assemble with  $\beta$  and  $\delta$  subunits, and are found extrasynaptically, mediating tonic inhibition [13]. The  $\delta$  subunit is found almost exclusively in the extrasynaptic domain and forms  $\alpha 4\beta\delta$  or  $\alpha 6\beta\delta$  receptors in the cortex and cerebellum, respectively [2]. However, it has also been suggested that  $\delta$  can co-assemble with  $\alpha 1$  subunits in hippocampal interneurons [14,15].

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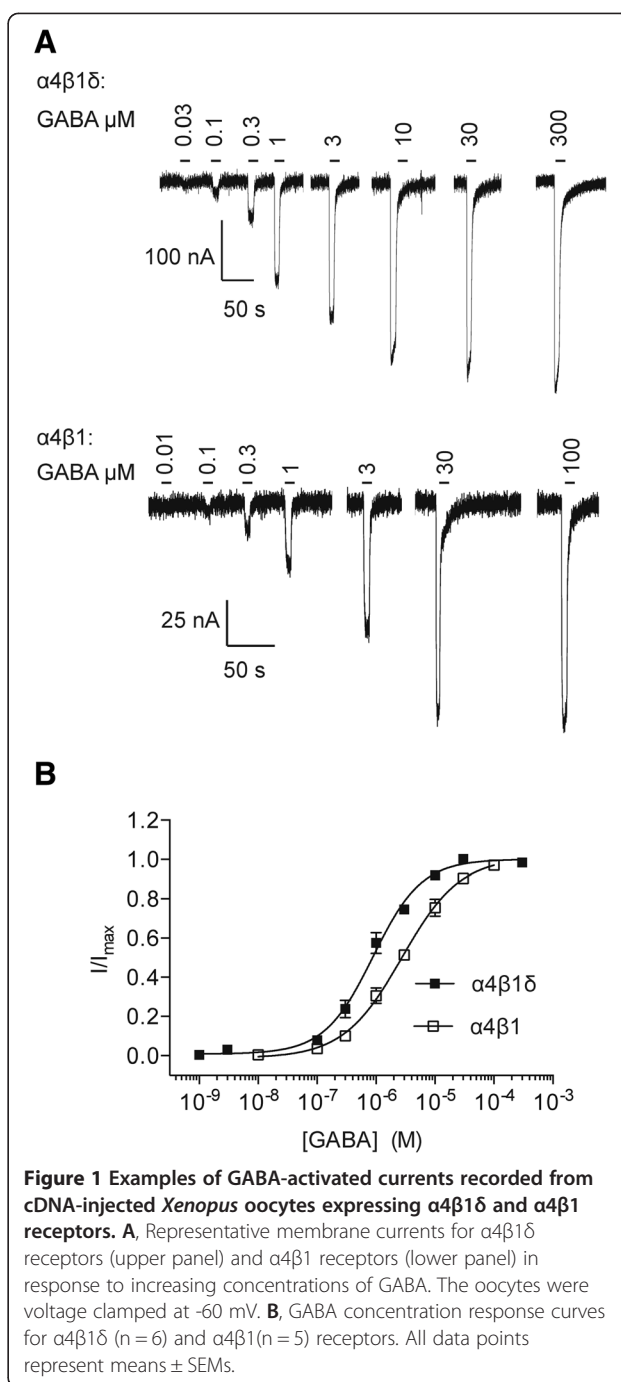
The existence of  $\alpha 4\beta 2/3\delta$  receptors in brain regions such as the hippocampal dentate gyrus is well established, but there is a paucity of data which characterises the potential native expression of the  $\alpha 4\beta 1\delta$  receptor [2]. This is largely due to the limited number of  $\beta 1$ -subtype-selective compounds available [16], and the fact that no  $\beta 1$  subunit knock-out mouse has been generated. As a consequence, most previous studies investigating  $\alpha 4\beta \delta$  receptors have focused on those subtypes containing the  $\beta 2$  or  $\beta 3$  subunits as these are confirmed to be native receptor combinations [2,17,18]. However, co-regulation of the expression levels of  $\alpha 4$ ,  $\beta 1$  and  $\delta$  subunits in, for example, the periaqueductal grey matter throughout the female oestrous cycle has been reported, suggesting that  $\alpha 4\beta 1\delta$  receptors may perform a hitherto overlooked important physiological role [19]. Also, experimental evidence for the co-expression of  $\alpha 4$ ,  $\beta 1$  and  $\delta$  subunits at extrasynaptic sites exists for pyramidal hippocampal neurons [20]. Furthermore, recent studies have emphasized the importance of  $\beta 1$ -containing receptors in relation to sleep control [21] and ataxia [22], and polymorphic mutants of the  $\beta 1$  subunit are thought to be important in determining the extent of alcohol consumption [23]. Therefore, since  $\alpha 4$ ,  $\beta 1$  and  $\delta$  subunits are co-expressed in several brain regions [24], the possibility exists that these subunits can co-assemble into native functional receptors, most likely at extrasynaptic sites, raising the prospect that such a receptor subtype may be important for neuronal physiology.

Few studies have investigated the properties of either human [25-27] or murine [23,28] recombinant  $\alpha 4\beta 1\delta$  receptors, and none of these have fully addressed the characterisation of classic GABA<sub>A</sub> receptor ligands. In these studies, interesting pharmacological differences between murine and human  $\alpha 4\beta 1\delta$  receptors are manifest: human receptors expressed in *Xenopus* oocytes [25] are constitutively active and display a GABA EC<sub>50</sub> in the mid-nanomolar range whereas the rat/mouse receptor expressed in oocytes or HEK293 cells display low-micromolar sensitivity towards activation by GABA [23,28]. To address this apparent species-dependent pharmacology and to address the effects of commonly used GABA receptor ligands, we report the pharmacological profiles for several GABA<sub>A</sub> receptor ligands at recombinant murine  $\alpha 4\beta 1$  and  $\alpha 4\beta 1\delta$  receptors expressed in *Xenopus laevis* oocytes.

## Results

### GABA concentration response relationships for $\alpha 4\beta 1$ and $\alpha 4\beta 1\delta$ receptors

GABA concentration response curves were generated for  $\alpha 4\beta 1$  and  $\alpha 4\beta 1\delta$  receptors to evaluate GABA potency at these receptors. Oocytes were challenged with increasing concentrations of GABA (Figure 1A). Depending upon expression levels, maximal currents to



**Figure 1** Examples of GABA-activated currents recorded from cDNA-injected *Xenopus* oocytes expressing  $\alpha 4\beta 1\delta$  and  $\alpha 4\beta 1$  receptors. **A**, Representative membrane currents for  $\alpha 4\beta 1\delta$  receptors (upper panel) and  $\alpha 4\beta 1$  receptors (lower panel) in response to increasing concentrations of GABA. The oocytes were voltage clamped at -60 mV. **B**, GABA concentration response curves for  $\alpha 4\beta 1\delta$  (n = 6) and  $\alpha 4\beta 1$  (n = 5) receptors. All data points represent means  $\pm$  SEMs.

GABA ranged from 100 to 3000 nA for cells expressing  $\alpha 4\beta 1$  receptors and from 300 to 3000 nA for cells expressing  $\alpha 4\beta 1\delta$  receptors. The holding currents were between 0 and -40 nA and the receptors were not constitutively active. The GABA EC<sub>50</sub> values were 0.89  $\mu\text{M}$  for  $\alpha 4\beta 1\delta$  receptors and 2.7  $\mu\text{M}$  for  $\alpha 4\beta 1$  receptors (Figure 1B, Table 1). GABA concentration-response data were fitted as monophasic curves, with Hill coefficients of 1.1 and 0.87 for  $\alpha 4\beta 1\delta$  and  $\alpha 4\beta 1$  receptors, respectively (Table 1).

**Table 1 Pharmacological profiles of GABA<sub>A</sub> ligands at  $\alpha 4\beta 1$  and  $\alpha 4\beta 1\delta$  receptors**

	EC <sub>50</sub> ( $\mu$ M) (pEC <sub>50</sub> $\pm$ SEM)	IC <sub>50</sub> ( $\mu$ M) (pIC <sub>50</sub> $\pm$ SEM)	n <sub>H</sub> ( $\pm$ SEM)	N
<b>GABA</b>				
$\alpha 4\beta 1\delta$	0.89 (6.05 $\pm$ 0.05)		1.1 $\pm$ 0.11	6
$\alpha 4\beta 1$	2.7 (5.56 $\pm$ 0.05)		0.87 $\pm$ 0.079	5
<b>Zn<sup>2+</sup></b>				
$\alpha 4\beta 1\delta$		3.3 (5.48 $\pm$ 0.03)	-0.75 $\pm$ 0.041	6
$\alpha 4\beta 1^a$		IC <sub>50A</sub> 0.00013 (9.87 $\pm$ 0.22)	ND	6
		IC <sub>50B</sub> 0.056 (7.25 $\pm$ 0.13)	-1.1 $\pm$ 0.13	
<b>Cu<sup>2+</sup></b>				
$\alpha 4\beta 1\delta$		0.82 (6.09 $\pm$ 0.04)	-1.2 $\pm$ 0.13	12
<b>SCS</b>				
$\alpha 4\beta 1\delta$		0.0042 (8.37 $\pm$ 0.25)		4

Potencies for GABA, Zn<sup>2+</sup>, Cu<sup>2+</sup> and SCS at  $\alpha 4\beta 1$  and  $\alpha 4\beta 1\delta$  receptors are listed. For Zn<sup>2+</sup> and Cu<sup>2+</sup>, inhibition was measured at the GABA EC<sub>75</sub>. For SCS, inhibition was measured at the GABA EC<sub>20</sub>. ND, not determined. <sup>a</sup> Data fit a two-site model (IC<sub>50A</sub> & IC<sub>50B</sub>) better than a one-site model as determined by an F-test ( $p < 0.0001$ ).

### Inhibition of GABA responses by divalent cations

Zn<sup>2+</sup> has been shown to be a useful pharmacological tool to distinguish  $\alpha\beta$  receptors from  $\alpha\beta\delta/\gamma$  receptors in recombinant expression systems, as  $\delta/\gamma$  subunit-containing GABA<sub>A</sub> receptors are less sensitive to inhibition by Zn<sup>2+</sup> compared to their  $\alpha\beta$  counterparts [29,30]. The increased potency at  $\alpha\beta$  receptors is attributed to the incorporation of a third  $\beta$  subunit that contains an important histidine residue for coordinating Zn<sup>2+</sup> ions at the top of channel lining M2 region [30,31].

We evaluated Zn<sup>2+</sup>-inhibition of the GABA response for both  $\alpha 4\beta 1$  and  $\alpha 4\beta 1\delta$  receptor isoforms to investigate whether Zn<sup>2+</sup> can distinguish between these receptor subtypes. Oocytes were pre-incubated for 1 min with various concentrations of Zn<sup>2+</sup> followed by co-application of Zn<sup>2+</sup> and GABA at an EC<sub>75</sub> (3  $\mu$ M for  $\alpha 4\beta 1\delta$  and 8  $\mu$ M for  $\alpha 4\beta 1$ ). As expected, Zn<sup>2+</sup> exhibited a higher potency at  $\alpha 4\beta 1$  than  $\alpha 4\beta 1\delta$  receptors. For  $\alpha 4\beta 1\delta$  receptors, Zn<sup>2+</sup> inhibited the GABA response with an IC<sub>50</sub> of 3.3  $\mu$ M (Figure 2A,B, Table 1). By contrast, for  $\alpha 4\beta 1$  receptors, biphasic inhibitory behaviour was observed with a high potency component (IC<sub>50A</sub> = 0.13 nM) accounting for ~25% of the total inhibition, and a more dominant lower potency component (IC<sub>50B</sub> = 56 nM) accounting for the remaining (~75%) inhibition (Figure 2A,B, Table 1).

Cu<sup>2+</sup> is another well-established inhibitor of GABA currents at  $\gamma$  subunit-containing GABA<sub>A</sub> receptors [32], but its inhibitory profile at  $\delta$  subunit-containing receptors has only recently been explored [33]. This revealed that Cu<sup>2+</sup> was far more potent as an inhibitor of steady-state GABA responses at  $\alpha 6\beta 3\delta$  receptors (IC<sub>50</sub> = 65 nM) compared to  $\alpha 1\beta 2\gamma 2$  receptors (IC<sub>50</sub> = 85  $\mu$ M) expressed in human embryonic kidney 293 (HEK293) cells [33]. To further

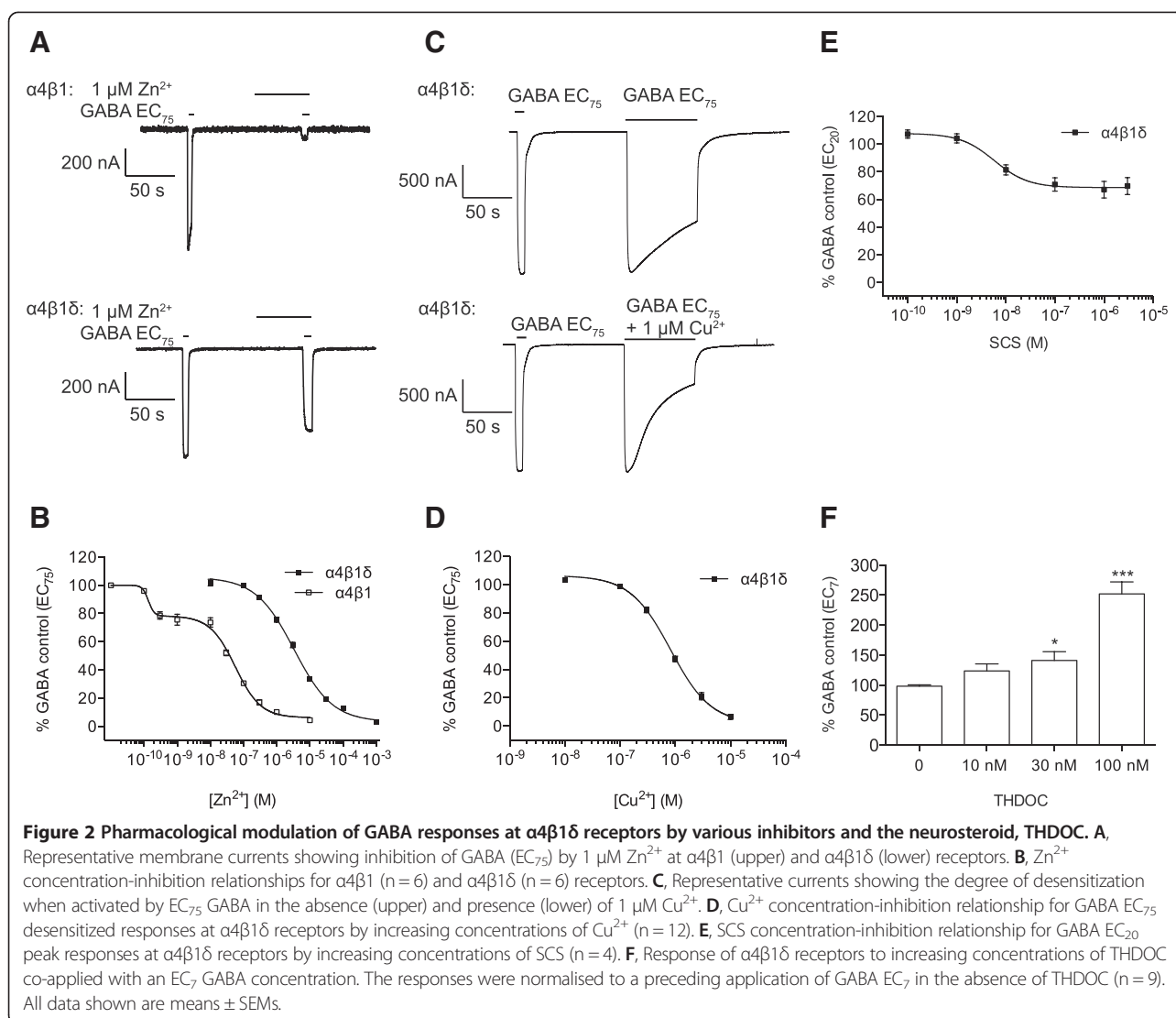
investigate the inhibitory selectivity of Cu<sup>2+</sup> at  $\alpha 4\beta 1\delta$  receptors, we co-applied increasing concentrations of Cu<sup>2+</sup> with GABA (EC<sub>75</sub>: 3  $\mu$ M) for 70 s, and measured the steady-state responses, reproducing the experimental conditions used by McGee et al. [33] to determine the IC<sub>50</sub> values. This response was taken as the fraction of the peak response of a previously-applied GABA concentration, and normalized to the steady-state response of GABA EC<sub>75</sub> in the absence Cu<sup>2+</sup> (Figure 2C). An almost complete inhibition of the  $\alpha 4\beta 1\delta$  GABA-activated response was observed with increasing concentrations of Cu<sup>2+</sup> (IC<sub>50</sub> = 0.82  $\mu$ M) (Figure 2D, Table 1).

### Inhibition by the $\beta 1$ subunit-selective blocker SCS

The salicylic acid derivative, SCS, is one of only a few compounds known to selectively target  $\beta 1$  subunit-containing GABA<sub>A</sub> receptors over  $\beta 2$ - and  $\beta 3$ -containing receptors [34]. SCS is reported to inhibit GABA-activated responses of  $\alpha 2\beta 1\gamma 2$ ,  $\alpha 2\beta 1\gamma 1$  and  $\alpha 2\beta 1\gamma 1\theta$  receptors, but the pharmacological profile of SCS at  $\alpha 4$  or  $\delta$ -containing receptors, has not been addressed. To examine the interaction of SCS with  $\alpha 4\beta 1\delta$  receptors, we evaluated the inhibition of the GABA EC<sub>20</sub> response following pre-application of the antagonist. In this instance, a low EC<sub>20</sub> concentration was chosen to enable a direct comparison between our study and that obtained previously [34]. SCS inhibited the GABA response with an IC<sub>50</sub> of 4.2 nM, but did not cause complete inhibition of the GABA current, attaining a steady-state level of inhibition at 67.2  $\pm$  3.2% of control (Figure 2E, Table 1).

### Neurosteroid potentiation at $\alpha 4\beta 1\delta$ receptors

Neurosteroids are potent modulators at most, if not all, GABA<sub>A</sub> receptor isoforms [35]. Some studies report



that neurosteroids are more potent at recombinant  $\delta$  subunit-containing receptors compared to their  $\gamma$  subunit-containing counterparts [17,36,37]. Accordingly, we evaluated the sensitivity of  $\alpha 4\beta 1\delta$  receptors to neurosteroid-induced potentiation. Increasing concentrations of THDOC were co-applied with an approximately  $EC_7$  GABA concentration. A significant increase in the GABA peak-response was recorded with THDOC concentrations of 30 and 100 nM ( $P < 0.05$ , one-way analysis of variance (ANOVA) with Dunnett's post test, Figure 2F).

## Discussion

In this study we investigated the pharmacological profiles of several GABA<sub>A</sub> receptor ligands at murine  $\alpha 4\beta 1\delta$  receptors. The GABA potency of  $0.89 \mu M$  ( $EC_{50}$ ) at  $\alpha 4\beta 1\delta$  receptors determined in this study is similar to that previously reported for the rat receptor ( $2.02 \mu M$ ) expressed in *Xenopus* oocytes [28], and the mouse/rat

receptor ( $1.99 \mu M$ ) expressed in HEK293 cells [23]. However, a much higher GABA potency ( $EC_{50}$  of 24 nM) has been reported for the human  $\alpha 4\beta 1\delta$  receptor expressed in *Xenopus* oocytes [25]. The GABA potency for human  $\alpha 4\beta 1$  ( $0.72 \mu M$ ) is reported to be somewhat lower [25] than the murine combination studied here. Furthermore, constitutive activity in the absence of GABA was reported for the human recombinant  $\alpha 4\beta 1\delta$  receptor [25], but this was not evident in our studies with the murine equivalent. From these observations it is possible that differences in constitutive activity may explain the considerable differences in GABA potency reported between species observed by others and in this study. However, several other factors may also influence the observed potency shift, such as differences in relative subunit expression levels which may significantly impact on the final receptor stoichiometry. In particular,  $\delta$ -containing receptors are prone to such variations in

stoichiometry. This is evident from studies in which variations in the amount of RNA injected into *Xenopus* oocytes apparently yield different receptor populations [38]; likewise, concatemeric receptors also demonstrate that alternative assemblies of functional  $\delta$ -containing receptors are possible [12,39]; and from reports that both high and low affinity conformations of the related  $\alpha 4\beta 3\delta$  receptor apparently exist [25,40].

In this study we report the first characterisation of specific GABA antagonists at  $\alpha 4\beta 1\delta$  receptors. The approximately 60-fold potency shift in the  $IC_{50}$  values for  $Zn^{2+}$  from low micromolar to mid nanomolar, when comparing  $\alpha 4\beta 1\delta$  and  $\alpha 4\beta 1$ , is consistent with prior observations made for  $\alpha 4\beta 3\delta$  and  $\alpha 4\beta 3$  receptors [29]. Our determination of monophasic  $Zn^{2+}$  potency suggests it is highly unlikely that there is any contamination with binary  $\alpha 4\beta 1$  receptors in our  $\alpha 4\beta 1\delta$ -expressing oocytes. Also, the inability of  $Zn^{2+}$  to inhibit the response below the holding current baseline corroborates the absence of any constitutive activity caused by homomeric  $\beta 1$  receptors [41].

It has previously been suggested that the stoichiometry of binary GABA<sub>A</sub> receptors containing only  $\alpha$  and  $\beta$  subunits is dependent on the type of  $\alpha$  and  $\beta$  subunit present, with a  $3\alpha:2\beta$  stoichiometry predominating for  $\alpha 1\beta 2$  receptors, but a  $2\alpha:3\beta$  stoichiometry preferred for  $\alpha 4\beta 2$  receptors [11]. The biphasic inhibitory behaviour of  $Zn^{2+}$  in our study would suggest the presence of a mixed GABA<sub>A</sub> receptor population, perhaps with both  $3\alpha:2\beta$  and  $2\alpha:3\beta$  stoichiometries expressed in the oocyte. This conclusion is rationalized by the fact that an additional  $\beta$  subunit will contribute another H267 residue, known to be critical for  $Zn^{2+}$  inhibition [31], which may underpin the higher sensitivity component to  $Zn^{2+}$  in the biphasic inhibition curves.

This study also evaluated the pharmacology of additional compounds at  $\alpha 4\beta 1\delta$  known to interact with GABA<sub>A</sub> receptors. We have established that the pharmacological profile of the  $\beta 1$ -selective inhibitor SCS, at  $\alpha 4\beta 1\delta$  receptors, is in agreement with the values reported for  $\alpha 2\beta 1\gamma 2$  receptors when expressed in *Xenopus* oocytes ( $IC_{50} = 4.36$  nM and maximum inhibition of ~33% [34], suggesting that  $\alpha 4$  and  $\delta$  subunits do not strongly influence the molecular interaction with SCS.

Our data evaluating the inhibition of desensitized GABA responses by  $Cu^{2+}$  suggest that the maximal inhibition of  $Cu^{2+}$  is greater at  $\alpha 4\beta 1\delta$  (98% inhibition at 10  $\mu$ M: expressed in oocytes) than at  $\alpha 6\beta 3\delta$  receptors (expressed in HEK293) where the maximal inhibition of the steady-state response was reported to be ~68% [33]. Although this difference may conceivably be due to the different expression systems, it is equally likely that it reflects some subunit-selectivity ( $\alpha 4 > \alpha 6$ ) for  $Cu^{2+}$ .

As hypothesized, THDOC concentrations at 30 nM and 100 nM were able to potentiate low ( $EC_7$ ) GABA

concentrations, suggesting that  $\alpha 4\beta 1\delta$  receptors are likely to be modulated by physiologically relevant concentrations of neurosteroids, as akin to many other GABA<sub>A</sub> receptor subtypes [35]. This is unsurprising given that the highly conserved neurosteroid binding site for potentiating GABA responses is contained within the receptor  $\alpha$  subunit.

## Conclusions

In summary, we have extended the pharmacological profile of the  $\alpha 4\beta 1\delta$  receptor, a subtype that has been studied surprisingly little compared to its  $\beta 2$ - and  $\beta 3$ -containing counterparts, despite its presence in many significant brain areas. We report a high GABA potency ( $EC_{50}$  of 0.89  $\mu$ M), a weaker sensitivity to  $Zn^{2+}$  compared to  $\alpha 4\beta 1$  receptors, and a high sensitivity to SCS and THDOC.

The observed discrepancy in potency compared to the human ortholog highlights the need for caution when making comparisons of pharmacological parameters across species for this particular GABA receptor subtype. For example, in addition to the different GABA potencies, there are notable differences in constitutive activity for this receptor isoform. Whether these differences translate into the native behaviour of this receptor remains to be determined. Finally, the different expression pattern of the  $\beta 1$  subunit compared to  $\beta 2$  and  $\beta 3$  subunits [24] suggests that  $\beta 1$ -containing subtypes may offer novel therapeutic targets and opportunities, and emphasizes a greater need for subtype-selective ligands and/or genetically modified mice to better understand the native function and pharmacology of this GABA<sub>A</sub> receptor isoform. Indeed recent findings suggest a clinically relevant role for  $\beta 1$ -containing extrasynaptic GABA<sub>A</sub> receptors in the nucleus accumbens mediating addictive behaviours related to excessive alcohol intake [23].

## Methods

Mouse  $\alpha 4$  and  $\beta 1$  and rat  $\delta$  cDNA were subcloned into a pRK5 expression vector using methods previously described [42].  $\gamma$ -Aminobutyric acid (GABA),  $CuCl_2$  and tetrahydro-deoxycorticosterone (THDOC) were purchased from Sigma (Dorset, UK),  $ZnCl_2$  from BDP Biochemical (Poole, UK) and salicylidine salicylhydrazide (SCS) from Tocris (Bristol, UK).

## Expression of GABA subunits

Oocytes from *Xenopus laevis* toads were prepared as described [43]. Briefly, ovaries were removed using procedures approved by the UK Animals (Scientific Procedures) Act 1986 and local (UCL) ethics committee. To obtain free oocytes, ovarian lobes were cut into pieces and incubated in OR2 (85 mM NaCl, 5 mM HEPES, 1 mM  $MgCl_2$ , pH adjusted to 7.6 with KOH) supplemented with 2-

2.5 mg/mL collagenase type 1 (Lorne Laboratories, Danehill, UK) for 3-4 hrs. Oocytes were washed several times in OR2 and subsequently in Barth's solution (88 mM NaCl, 1 mM KCl, 0.33 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.41 mM CaCl<sub>2</sub>, 0.82 mM MgSO<sub>4</sub>, 2.4 mM NaHCO<sub>3</sub>, 10 mM HEPES, pH adjusted to 7.6 with NaOH) to isolate cells and remove follicular membranes. After preparation, the oocytes were injected with 27 or 55 nL of 30 ng/μL cDNA in a ratio of 1:1 (α4β1) or 1:1.5 (α4β1δ). Oocytes were subsequently maintained at 18°C in Barth's solution supplemented with gentamycin (Invitrogen, Paisley, UK).

### Electrophysiological recordings

Recordings were performed at room temperature 2-4 days (α4β1) or 3-6 days (α4β1δ) post injection using two-electrode voltage-clamp (Axoclamp 2B amplifier, Digidata 1322A interface and pClamp 8 (Molecular Devices, Wokingham, UK)). Electrodes were filled with 3 M KCl and had resistances of 0.5 to 3 MΩ. The oocytes were clamped at a holding potential of -60 mV and were perfused with buffer (100 mM NaCl, 2 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, pH adjusted to 7.4 with NaOH) throughout the recordings. All currents were adjusted when necessary for voltage drop using equation 1:

$$I = I_{measured} \cdot \frac{(V_h - V_{rev})}{(V_h - V_{rev}) + \Delta V} \quad (1)$$

Where  $I_{measured}$  is the observed current,  $V_h$  is the holding potential (mV),  $V_{rev}$  is the Cl<sup>-</sup> reversal potential for oocytes (usually ~ -30 mV), and  $\Delta V$  is the change in the holding potential (mV) due to loss of clamp during at the peak of large GABA-evoked currents. Drugs were applied using a fast application system coupled to a small bath volume (~1 ml), enabling drug onset times of ~ 10 ms.

### Generation of concentration response curves

Agonist concentration response curves were generated by measuring peak responses normalized to a standard concentration of GABA applied between each or every second application (typically an EC<sub>10-20</sub> concentration). To pool data from different oocytes, the data were fitted and normalized to the calculated peak of the curve using GraphPad Prism (version 6.0) according to equation 2;

$$\frac{I}{I_{max}} = \frac{1}{1 + \left(\frac{EC_{50}}{[GABA]}\right)^n} \quad (2)$$

where  $I$  and  $I_{max}$  are the GABA activated current and maximal GABA current, respectively, and the EC<sub>50</sub> is the GABA concentration inducing a half-maximal response and  $n$  is the Hill slope.

Zn<sup>2+</sup> and SCS inhibition curves were generated by pre-application (for 60 s) of the inhibitor followed by co-applications of the inhibitor and GABA. For each co-application, the peak-response was measured. For Cu<sup>2+</sup>-inhibition curves, Cu<sup>2+</sup> and GABA were co-applied and the steady-state response after 70 s was measured and normalized to a preceding GABA-application. All monophasic inhibition-curves were fitted to a modified version of equation 2. The biphasic Zn<sup>2+</sup>-inhibition curve was fitted to equation 3:

$$I_N / I_N = [1 - i B^n_H / (B^n_H + (IC_{50A}^n)_H)] - j B^n_H / (B^n_H + (IC_{50B}^n)_H)] \quad (3)$$

where  $I'_N$  and  $I_N$  represent the normalised GABA-induced current in the presence and absence of Zn<sup>2+</sup> at a concentration ( $B$ ) respectively.  $(IC_{50})_{A/B}$  define the concentrations of Zn<sup>2+</sup> producing 50% inhibition of the GABA-induced current and  $i$  and  $j$  represent weighting factors for the components A and B, where  $i + j = 1$ .

Potentiation by the neurosteroid THDOC was measured by co-application of GABA (EC<sub>7</sub>) and THDOC until a peak response was attained. Normalized responses were pooled and plotted as mean ± SEM from at least four different oocytes taken from at least two different *Xenopus* toads. For statistical evaluation, a repeated measures ANOVA with Dunnett's post test was used. The significance level was set at \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001. For inhibition curves, an F-test was performed to determine whether the data was either monophasic or biphasic.

### Abbreviations

GABA: γ-aminobutyric acid; HEK293: Human embryonic kidney 293.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

ISV, PW and TGS designed the experiments. ISV conducted the experiments. ISV, PW and TGS analysed and interpreted the data. ISV, PW and TGS wrote or contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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