BJP British Journal of Pharmacology

DOI:10.1111/bph.12001
www.brjpharmacol.org

RESEARCH PAPER

A study of subunit selectivity, mechanism and site of action of the delta selective compound 2 (DS2) at human recombinant and rodent native $GABA_A$ receptors

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Keywords
$GABA_A$ receptor; DS2; δ-subunit; α4-subunit; site of interaction; brain-slice electrophysiology

Received
13 January 2012
Revised
18 July 2012
Accepted
23 August 2012

BACKGROUND AND PURPOSE

Most $GABA_A$ receptor subtypes comprise 2α, 2β and 1γ subunit, although for some isoforms, a δ replaces a γ-subunit. Extrasynaptic δ-$GABA_A$ receptors are important therapeutic targets, but there are few suitable pharmacological tools. We profiled DS2, the purported positive allosteric modulator (PAM) of δ-$GABA_A$ receptors to better understand subtype selectivity, mechanism/site of action and activity at native δ-$GABA_A$ receptors.

EXPERIMENTAL APPROACH

Subunit specificity of DS2 was determined using electrophysiological recordings of *Xenopus laevis* oocytes expressing human recombinant $GABA_A$ receptor isoforms. Effects of DS2 on GABA concentration–response curves were assessed to define mechanisms of action. Radioligand binding and electrophysiology utilising mutant receptors and pharmacology were used to define site of action. Using brain-slice electrophysiology, we assessed the influence of DS2 on thalamic inhibition in wild-type and $\delta^{0/0}$ mice.

KEY RESULTS

Actions of DS2 were primarily determined by the δ-subunit but were additionally influenced by the α, but not the β, subunit (α4/6βxδ > α1βxδ >> γ2-$GABA_A$ receptors > α4β3). For δ-$GABA_A$ receptors, DS2 enhanced maximum responses to GABA, with minimal influence on GABA potency. (iii) DS2 did not act *via* the orthosteric, or known modulatory sites on $GABA_A$ receptors. (iv) DS2 enhanced tonic currents of thalamocortical neurones from wild-type but not $\delta^{0/0}$ mice.

CONCLUSIONS AND IMPLICATIONS

DS2 is the first PAM selective for α4/6βxδ receptors, providing a novel tool to investigate extrasynaptic δ-$GABA_A$ receptors. The effects of DS2 are mediated by an unknown site leading to $GABA_A$ receptor isoform selectivity.

Abbreviations

DS1, delta selective compound 1; DS2, delta selective compound 2; EC_x, effective concentration giving the activation degree of X; $GABA_AR$, $GABA_A$ receptor; NAM, negative allosteric modulator; PAM, positive allosteric modulator; VB neurons, ventrobasal thalamic neurons

Introduction

The $GABA_A$ receptor is the major inhibitory receptor in the mammalian brain. When the neurotransmitter GABA binds to and activates the receptor, an associated conformational change of the protein permits chloride ions to pass through the receptor pore, thereby leading in most instances to neuronal hyperpolarization. $GABA_A$ receptors are pentameric proteins made from the assembly of homologous subunits of various types (α1–6, β1–3, γ1–3, δ, ε, θ and π) (Sieghart, 1995; Barnard *et al.*, 1998; receptor nomenclature follows Alexander *et al.*, 2011). The majority of $GABA_A$ receptors have the general stochiometry of 2α, 2β and 1γ subunit. However, a subpopulation of receptors is composed of 2α, 2β and 1δ subunit, with the δ-subunit replacing the γ-subunit. $GABA_A$ receptors incorporating the δ-subunit are primarily found in peri- or extrasynaptic locations where they are subject to activation by low ambient GABA concentrations (Belelli *et al.*, 2009). Indeed, δ-$GABA_A$ receptors are highly sensitive to GABA and exhibit little desensitization (Nusser *et al.*, 1998; Farrant and Nusser, 2005; Houston *et al.*, 2009; Bright *et al.*, 2011). GABA 'spill-over' from the synapse reaching a concentration in the low μM range (Mody, 2005) is believed to contribute to the tonic activation of extrasynaptic receptors. This suggestion has been supported by electrophysiological studies using brain slice preparations, where in certain neurones the $GABA_A$ receptor antagonists bicuculline, gabazine and picrotoxin, inhibit the basal current (Brickley *et al.*, 1996; 1999; Jia *et al.*, 2005; Chandra *et al.*, 2006).

Three δ-$GABA_A$ receptor isoforms have been identified *in vivo*, namely α4β2/3δ receptors expressed in thalamic relay neurons and dentate gyrus granule cells (Porcello *et al.*, 2003; Belelli *et al.*, 2005; Chandra *et al.*, 2006), α6β2/3δ receptors expressed in cerebellar granule cells (Nusser *et al.*, 1998) and a population of α1β2/3δ receptors, proposed to occur in certain hippocampal interneurons (Sun *et al.*, 2004; Mangan *et al.*, 2005). Mice with a genetic deletion of the δ-subunit have provided an insight to the physiological relevance of these δ-$GABA_A$Rs, knowledge that has been supplemented with a phenotypic analysis of α4 and α6 'knock-out' mice, the two α-subunits primarily associated with the δ-subunit. For example, $\delta^{0/0}$ mice are more susceptible to seizures (Spigelman *et al.*, 2002), have enhanced trace fear conditioning (Wiltgen *et al.*, 2005) and have an altered response in phase 2 of the formalin-induced nociception test (Bonin *et al.*, 2011). Further tonic currents in the thalamus and dentate gyrus of $\alpha4^{0/0}$ mice (Chandra *et al.*, 2006) and in cerebellar granule cells in $\alpha6^{0/0}$ mice (Brickley *et al.*, 2001) are significantly reduced.

From a therapeutic perspective, δ-containing receptors are emerging as a potentially important pharmacological target. Importantly, δ-containing receptors are not modulated by classical $GABA_A$ receptor modulators acting *via* the benzodiazepine binding site located between the γ- and α-subunits, although other known modulators of $GABA_A$ receptors such as neurosteroids, etomidate and barbiturates enhance the function of δ-$GABA_A$ receptors (Belelli *et al.*, 2005). However, these latter modulators exhibit little selectivity for δ- over γ-containing receptors (Belelli *et al.*, 2002; Brown *et al.*, 2002; Wohlfarth *et al.*, 2002; Zheleznova *et al.*, 2008), precluding any pharmacological verification of the physiological or pathophysiological role of δ-$GABA_A$ receptors. One exception is the relatively δ-preferring agonist THIP, or gaboxadol, which has provided some insight on the role of δ-$GABA_A$ receptors in sleep regulation. For example, the effect of gaboxadol to enhance tonic inhibition in thalamocortical neurons and to produce hypnosis and ataxia were blunted in $\delta^{0/0}$ mice (Herd *et al.*, 2009). Moreover, in $\alpha4^{0/0}$ mice (α4 is a δ-subunit preferring partner in certain neurons), the behavioural effects of gaboxadol such as sedation, ataxia and analgesia were curtailed (Chandra *et al.*, 2006). Based on the preclinical data on gaboxadol demonstrating a hypnotic profile in rats (Thakkar *et al.*, 2008) and that the effects of the drug on EEG were blunted in $\delta^{0/0}$ mice (Winsky-Sommerer *et al.*, 2007), it was pursued as a novel treatment for insomnia, reaching phase III clinical development (Wafford and Ebert, 2006; Roth *et al.*, 2010). Given the literature indicating a role for δ-$GABA_A$ receptors in female stress disorders (Maguire and Mody, 2007; Smith *et al.*, 2007), epilepsy (Mihalek *et al.*, 1999), pain (Peng *et al.*, 2009; Bonin *et al.*, 2011), post-traumatic stress disorder (Wiltgen *et al.*, 2005; Pibiri *et al.*, 2008), schizophrenia (Marx *et al.*, 2006), autism (Olmos-Serrano *et al.*, 2011), major depression (Holm *et al.*, 2010) and potentially alcoholism (Enoch, 2008; Rewal *et al.*, 2009), the need for δ-selective tools is clear. Furthermore, it should not be overlooked that a major swathe of the literature, only partly cited above, alluding to the potential therapeutic relevance of δ-$GABA_A$ receptors has been based on studies with neurosteroids, which are not completely selective for these extrasynaptic receptors (Belelli *et al.*, 2009). Therefore, a drug that is truly selective for δ-$GABA_A$ receptors would confirm and expand on the extant literature obtained with mostly non-selective drugs.

Few positive allosteric modulators (PAMs) selective for δ-$GABA_A$ receptors have been reported, and no selective negative allosteric modulators (NAMs) are known. The imidazopyridine DS2 is a functionally selective α4β3δ PAM, relative to its actions at α4β3γ2 and α1β3γ2 receptors, with clear effects on the tonic current of thalamic ventrobasal (VB) neurons mediated by α4β2δ receptors (Wafford *et al.*, 2009). Recently, the triamino-benzene compound AA29504, a retigabine analogue, was described as a functionally selective α4β3δ PAM, albeit the lack of full concentration–response analysis at δ- and γ-containing $GABA_A$ receptors precludes definitive conclusions (Hoestgaard-Jensen *et al.*, 2010). Nonetheless, AA29504 augments the effects of gaboxadol in cortical brain slices, enters the brain and is effective in some *in vivo* models. JM-11-43A, a dihydropyrimidinone, has also been described as a selective α4β3δ PAM, but as with AA29504, this drug appears to exhibit limited selectivity (Lewis *et al.*, 2010).

In the current study, we have explored in detail the properties of DS2 as a δ-$GABA_A$ receptor-selective allosteric modulator, employing the *Xenopus laevis* oocyte expression system to conduct a comprehensive electrophysiological analysis of the actions of DS2 acting at human recombinant $GABA_A$ receptors incorporating α1–6 subunits, combined with the relevant β, γ- and δ-subunits. We demonstrate the necessity of the δ-subunit for efficacy at α4-subunit-containing receptors and analyse the influence of different β-subunits on the

actions of DS2. We also provide a detailed mechanistic understanding of the effects of DS2. To determine the site of action of DS2, we have utilized both radioligand binding and electrophysiological methods employing mutant receptor constructs as necessary. Finally, we have demonstrated that the effect of DS2 at native receptors is mediated by δ-$GABA_A$ receptors by comparing its modulation of thalamic inhibition in wild-type and $\delta^{0/0}$ mice.

Methods

Cloning of $GABA_A$ receptor cDNAs

The cDNAs for human α1–6, β2–3 and γ2S-subunits were cloned as described previously (Mirza *et al.*, 2008). The cDNAs for the human β1- and δ-subunit were cloned from human hippocampus poly(A^+) mRNA (Clontech, Mountain View, CA) using RT-PCR. First-strand cDNA was obtained using oligo(dT) primer and Moloney murine leukaemia virus reverse transcriptase (GE Healthcare Life Sciences, UK) and full-length cDNAs were amplified using Expand HF polymerase (Roche Diagnostics, Basel, Switzerland) and gene-specific primer sets (MWG Biotech, High Point, NC). Twenty-five PCR cycles were performed using the following conditions: 94°C, 60 s; 55°C, 60 s; 72°C, 120 s, followed by 72°C, 10 min using a Robocycler (Stratagene, La Jolla, CA). The amplified product was cloned into pSwas [derived from pZErO-1 (Life Technologies, Invitrogen, Naerum, Denmark)]. Clones were sequenced bidirectionally and subcloned into the pNS3z or pNS3h vector derived from pcDNA3 (Invitrogen).

The $GABA_A$ receptor β3N265M and $GABA_A$ receptor α4T235W, Q240W subunits were constructed in a mutagenesis reaction. Briefly, uracilated β3- and α4-subunit-containing plasmids were used as templates in a mutagenesis reaction, in which mutagenic oligonucleotides and T7 DNA polymerase were used to introduce mutations. An aliquot of the mutagenesis reaction was then transformed into *Escherichia coli* XL1-Blue cells. Mutated plasmids were identified by the introduction or elimination of restriction sites.

The mutagenic oligonucleotides were β3N265M: GACAA TGACAACCATCAtgACCCACCTTCGGGAGA and α4T235W, Q240W: ATATTCCGTGCATTATGtggGTGATctTaagTtggGTTT CATTTTGGATAAA.

All mutated constructs were verified by sequencing.

Cell culture and stable transfections

Stably transfected HEK-293 (ATCC1573) cell lines were established as described (Mirza *et al.*, 2008) and maintained in DMEM with 10 mM HEPES and 2 mM Glutamax supplemented with 10% FBS. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air and passaged twice a week.

[^{3}H] muscimol, [^{3}H]Flumazenil and [^{3}H]Ro15-4513 binding studies

[^{3}H]Flumazenil and [^{3}H]Ro15-4513 binding studies were performed as described previously (Mirza *et al.*, 2008). [^{3}H]muscimol binding was performed using aliquots of membranes (native tissue or cell lines) resuspended in Tris–citrate buffer (50 mM, pH 7.1) and centrifuged for 10 min at 22 000× *g* at 4°C. The pellet was resuspended to give a protein concentration of 100–200 μg·mL^{-1}. Binding was performed in triplicate using [^{3}H]muscimol (25.5 Ci·$mmol^{-1}$; PerkinElmer, Waltham, MA) in a final volume of 550 μL containing 50–100 μg of protein, and non-specific binding was determined in the presence of 100 μM GABA (SIGMA Aldrich, St. Louis, MO). Samples were incubated at 4°C for 40 min, and labelled membranes were harvested using rapid filtration over GF/C filters (Whatman, Maidstone, UK). The filters were washed with 2 × 5-mL Tris–citrate buffer, and the amount of radioactivity was determined by liquid scintillation counting using a Tri-Carb counter (PerkinElmer).

Preparation of cRNA

Plasmids were linearized using a unique downstream polylinker enzyme (*Not*I, *Xho*I or *Xba*I) for cRNA production. cRNA was prepared and capped using the mMESSAGE mMACHINE T7 Transcription kit (Ambion, Austin, TX) and was purified using the RNeasy mini kit (QIAGEN, Hilden, Germany).

Isolation of X. laevis *oocytes*

X. laevis (Nasco, Fort Atkinson, WIUSA) oocytes were isolated as described previously (Mirza *et al.*, 2008). Oocytes were injected with 25 to 50 nL of cRNA mixtures of $GABA_A$R subunits αx : βx : δ/γ2S in the ratios 1:1:2 unless otherwise stated or 1:1:5 for α1 : β2 : δ using a Pico Pump (WPI, Sarasota, FL). Oocytes were maintained at 18°C in modified Barth's solution for up to 7 days after injection.

Oocyte electrophysiology

Electrophysiological studies using *X. laevis* oocytes were performed using the two-electrode voltage-clamp technique (Mirza *et al.*, 2008). Briefly, GABA was dissolved in OR2 buffer (composition, in mM: 90 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 5 HEPES; pH adjusted to 7.4) in a concentration known to give rise to $EC_{5\text{-}20}$ elicited currents for α1–3,5+β2γ2 receptors (0.5–3 μM) and $EC_{20\text{-}50}$ for α4β2/3δ (0.01–1 μM) receptors. This solution was used to establish control responses and for dissolving the compounds to test in the experiment. The modulatory effects of DS2 were calculated by comparing the trace obtained in the presence of DS2 to the control trace, where a doubling of the current equals a 100% increase.

Brain slice electrophysiology

All animal care and experimental procedures complied with the UK Government Animals (Scientific Procedures) Act 1986 and were approved by the 'University of Dundee Ethics Committee'. Studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). A total of 25 mice were used in the work described here. WT and $\delta^{0/0}$ mice were of a C57BL6 background and were bred at the University of Dundee.

Mice were killed by cervical dislocation in accordance with schedule 1 of the UK Government Animals (Scientific Procedures) Act 1986. Thalamic slices were prepared from mice of either sex (P18-24) as previously described (Belelli *et al.*, 2005). The brain was rapidly removed and placed in oxygenated ice-cold artificial cerebrospinal fluid (aCSF)

solution containing (in mM) 225 sucrose, 2.95 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 0.5 $CaCl_2$, 10 $MgSO_4$, 10 glucose, (pH of 7.4; 330–340 mOsm). The tissue was maintained in ice-cold aCSF from which horizontal 300–350 μm slices were prepared using a Vibratome (Intracel, Royston, Herts., UK). Slices were transferred to an incubation chamber containing oxygenated, extracellular solution [(ECS) in mM: 126 NaCl, 2.95 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 10 glucose and 2 $MgCl_2$ (pH 7.4; 300–310 mOsm)] for ≥1 h prior to recording. Patch pipettes (open tip resistances of 3–5 MΩ) were constructed using thick-walled borosilicate glass (Garner Glass Company, Claremont, CA) and filled with an intracellular solution that contained (in mM) 140 CsCl, 10 HEPES, 10 EGTA, 2 Mg-ATP, 1 $CaCl_2$, 5 QX-314 (pH 7.3 with CsOH, 300–305 mOsm). Whole-cell voltage-clamp recordings were performed at 35°C from thalamic VB neurons visually identified with an Olympus BX51 (Olympus, Southall, UK) microscope equipped with DIC/IR optics as previously described (Belelli *et al.*, 2005). Miniature inhibitory post-synaptic currents (mIPSCs) and tonic currents were recorded using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA) at a holding potential of –60 mV in ECS that, contained 2 mM kynurenic acid (Sigma-Aldrich-RBI, Dorset, UK) and 0.5 μM tetrodotoxin (TTX; TCS Biologicals Ltd, Buckingham, UK) to block ionotropic glutamate receptors and sodium-dependent action potentials respectively.

Data analysis

Data were recorded onto a digital audio tape using a Biologic DTR 1200 recorder and analysed offline using the Strathclyde Electrophysiology Software, WinEDR/WinWCP (J Dempster, University of Strathclyde, UK). Individual mIPSCs (with a rise time ≤1 ms) were detected as previously described (Belelli *et al.*, 2005). The peak amplitude, 10–90% rise time, time to decay from peak by 50% (T_{50}), 90% (T_{90}) and charge transfer (the area under the curve of each event) were determined for accepted events. The mIPSC frequency was determined over 10 s bins for 2 min. To calculate the time constant of mIPSC decay, a minimum of 50 accepted events were digitally averaged by alignment at the midpoint of the rising phase, and the mIPSC decay fitted by either monoexponential, or biexponential functions using the least squares method as previously described (Belelli *et al.*, 2005). Note the average mIPSC decay was always best fit with the sum of two exponential components. Thus, a weighted decay time constant (τ_w) was determined as described previously. The tonic current was calculated as the difference between the holding current before (determined by averaging the current for a minimum of 200 epochs of 25 ms that did not contain phasic events) and after application of (30 μM) bicuculline methobromide. All results are reported as the arithmetic mean ± SEM. Statistical significance of mean data was assessed with paired or unpaired Student's *t*-test as appropriate.

Materials

DS2 was synthesized at NeuroSearch A/S, Medicinal Chemistry Department. GABA, bicuculline, etomidate, allopregnanolone (5α-pregnan-3α-ol-20-one) and pentobarbital were purchased from Sigma Aldrich (St. Louis, MO).

Results

Effect of DS2 on α4-containing $GABA_A$ receptors (α4β3δ, α4β3γ2 and α4β3): the activity of DS2 is determined by the δ-subunit

We initially determined the importance of the δ-subunit by comparing the modulatory effects of DS2 on α4β3, α4β3δ and α4β3γ2 $GABA_A$ receptors.

α4β3δ, α4β3 and α4β3γ2 receptors were expressed in *X. laevis* oocytes, and GABA-evoked currents were measured 5–7, 2–4 and 2–4 days after RNA injection, respectively; preliminary studies allowed us to define these optimized timing conditions (data not shown). The receptors were activated by GABA and by gaboxadol, with the latter being a more efficacious agonist than GABA on both α4β3δ (205 ± 8% relative to GABA maximal efficacy, n = 6) and α4β3 receptors (185 ± 12% relative to GABA maximal efficacy, n = 5), compared with α4β3γ2 receptors (95 ± 8% relative to GABA maximal efficacy, n = 5). Furthermore, gaboxadol had approximately 5–10× greater potency than GABA at α4β3δ (EC_{50} = 24 ± 1 μM, n = 6) and α4β3 (EC_{50} = 12 ± 1 μM, n = 6) compared with its potency at α4β3γ2 receptors (EC_{50} = 116 ± 34, n = 5). The relative pharmacological profile of GABA and gaboxadol at these three receptors subtypes is in agreement with earlier findings in both a mouse L(tk^-) cell line (Brown *et al.*, 2002) and *Xenopus* oocytes (Storustovu and Ebert, 2006).

The modulatory effect of DS2 on α4-containing receptors was critically dependent on the presence of the δ-subunit in the receptor complex. A low concentration of GABA (10 nM) activated a relatively small inward current for the three α4-containing receptor subtypes. However, only GABA-evoked currents mediated by α4β3δ receptors were enhanced by the co-application of DS2 (0.1–10 μM; Figure 1A top left panel). DS2 (10 μM) produced a maximal 10-fold increase in such GABA-evoked currents with a calculated EC_{50} of 5.6 ± 1.5 μM (n = 11) – Figure 1A.

The very low concentration of GABA (10 nM) used in these experiments did not elicit a robust inward current from *Xenopus* oocytes expressing α4β3γ2 receptors. Therefore, the effect of DS2 was also investigated using 10 μM GABA to activate this receptor subtype. Under these conditions, 10 μM DS2 produced only a modest enhancement of the GABA-evoked current (Figure 1A, bottom left panel trace) being ≤20% of control compared with ≥10-fold increase at α4β3δ receptors. Thus, for α4-containing receptors, DS2 clearly discriminates between α4β3δ compared with α4β3γ2 and α4β3 receptors.

Effect of DS2 on additional $GABA_A$ receptors: DS2 is selective for extrasynaptic versus synaptic receptors

We next addressed whether DS2 modulated GABA-evoked currents at other γ2-containing $GABA_A$ receptors: namely, α1β2γ2, α2β2γ2, α3β2γ2 and α5β2γ2 receptors. With the exception of the α5β2γ2 receptor, these receptors are primarily expressed at the synapse (Fritschy and Mohler, 1995; Jacob *et al.*, 2008). For these experiments, all γ2-containing receptors were activated with a GABA concentration that

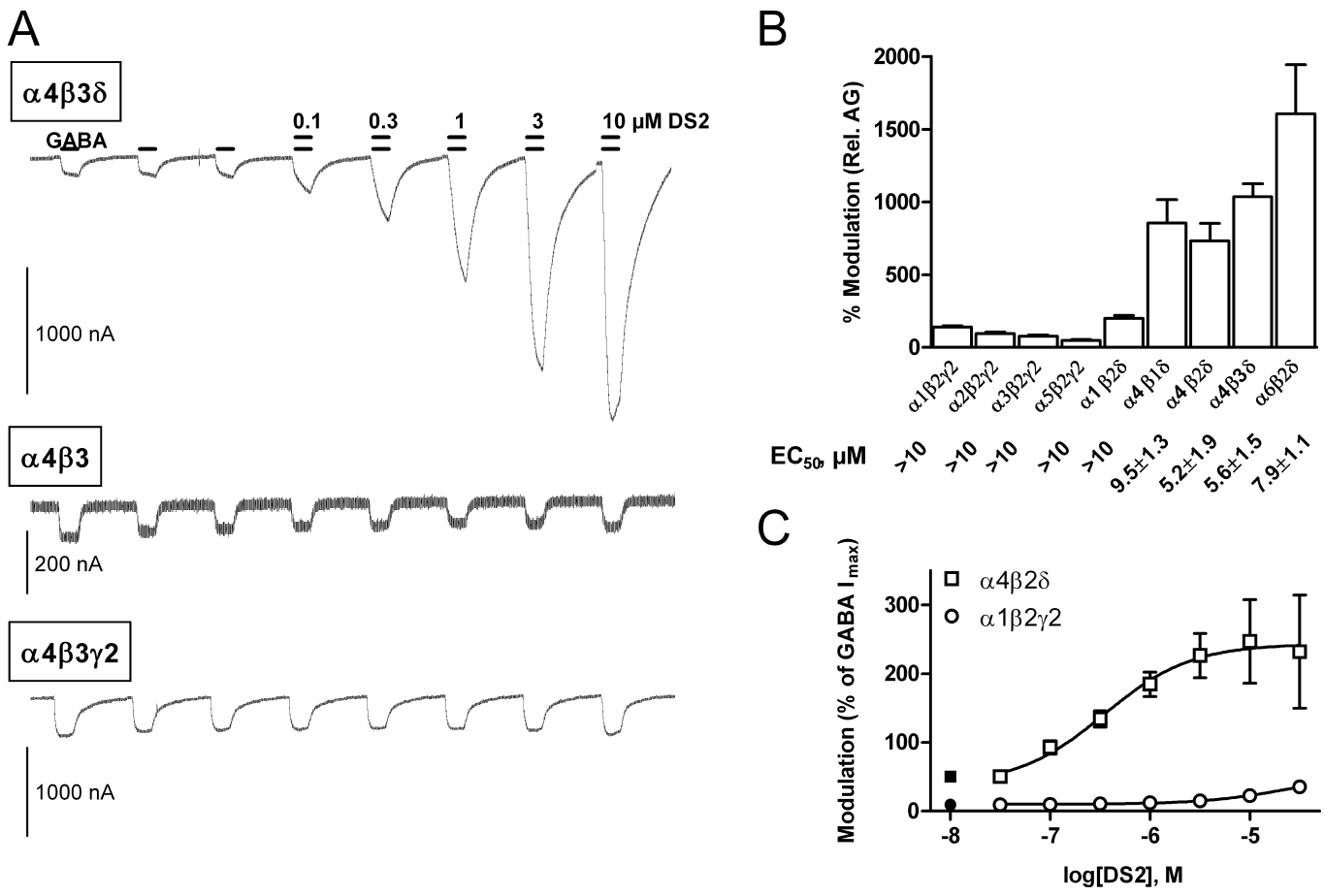


Figure 1

Selectivity of DS2 for various human recombinant $GABA_A$ receptors determined using two-electrode voltage-clamp electrophysiology. (A) Current traces recorded from *X laevis* oocytes expressing human recombinant α4β3δ, α4β3 or α4β3γ2 receptors. The bars above each trace represent a time period of 1 min, where the agonist/modulator was applied. Currents were elicited using either a low GABA concentration (0.01 μM for α4β3δ and α4β3 and 10 μM for α4β3γ2) or GABA in combination with the various concentrations of DS2 listed above each bar. There is an extra 1 min wash between each of the first five traces and 2 min wash between each of the last three to allow for full receptor activation on next drug application. Note that the traces are truncated and staggered, omitting the long wash periods – 90 s at lowest concentration, and up to 4 min at the highest concentration of DS2 – to focus on key experimental data only. A new response was elicited first on return to baseline. (B) Relative modulation of 10 μM DS2 on various $GABA_A$ receptor subtypes compared with a GABA response elicited using either GABA $EC_{5\text{-}20}$ for α1–5β2γ2 or $EC_{20\text{-}50}$ for α1β2δ, and α4β1–3δ and α6β2δ indicated as modulation relative to agonist (Modulation rel. AG) on the *y*-axis. EC_{50} values from full concentration–response curves given at the bottom of panel B are calculated using the Hill equation. *n*-numbers are $n = 5$ for α4β1δ, $n = 5$ for α4β2δ, $n = 11$ for α4β3δ and $n = 6$ for α6β2δ. (C) DS2 concentration–response curves related to the GABA I_{max} value for each *X. laevis* oocyte. GABA responses elicited with no DS2 present or at a given D2 concentration for α4β2δ [using GABA $EC_{20\text{-}50}$ (0.1–1 μM)] and with no DS2 present or at a given D2 concentration for α1β2γ2 [using GABA $EC_{5\text{-}20}$ (0.5–3 μM)].

elicited a current response corresponding to $EC_{5\text{-}20}$ and DS2 was co-applied with GABA in the concentration range of 100 nM to 10 μM. For these receptors, DS2 (0.1–1 μM) had no effect on the GABA-evoked current. At higher concentrations (3 and 10 μM), DS2 produced a modest but significant potentiation (range 47–139% of the submaximal GABA-evoked response) – see Figure 1B. However, these limited effects were at a low relative potency (EC_{50} values >10 μM for all γ2-containing receptors) compared with its EC_{50} (5.6 ± 1.5 μM) at α4β3δ – see summary EC_{50} values at bottom of Figure 1B.

Actions of DS2 are influenced by the α- but not the β-subunit

Whether the modulatory effect of DS2 with δ-$GABA_A$Rs was additionally influenced by the type of α- or β-subunit present in the receptor complex was addressed using the following receptor subtypes: α4β1δ, α4β2δ, α4β3δ, α1β2δ and α6β2δ. A maximal concentration of DS2 (10 μM) produced a large enhancement (7- to 20-fold) of the GABA (1 μM) evoked current mediated by all δ-containing receptors incorporating the α4- or the α6-subunit (Figure 1B) with calculated EC_{50} values of 5.2 ± 1.9 ($n = 5$) and 7.9 ± 1.1 ($n = 6$) μM for α4β2δ

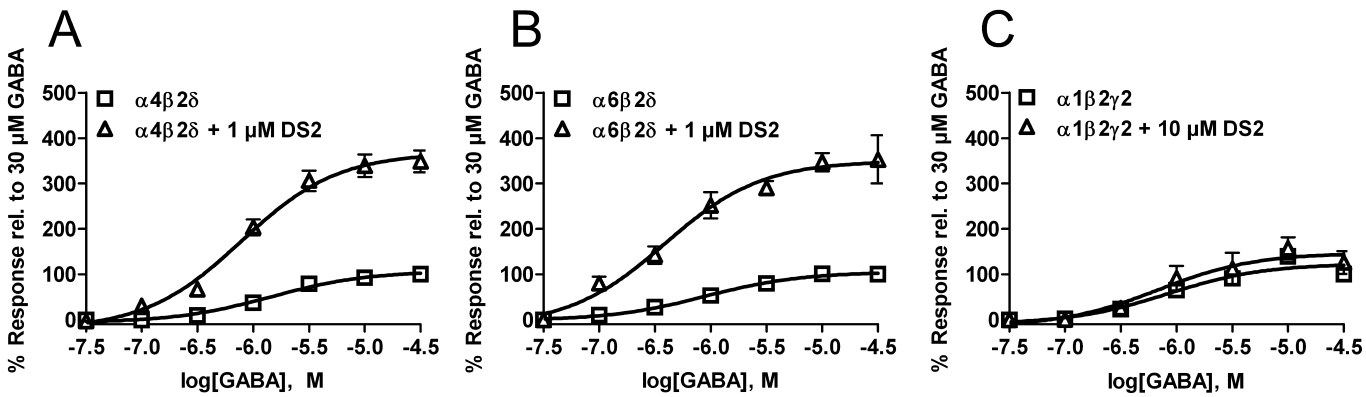


Figure 2

The influence of DS2 on the GABA concentration–response relationship for human recombinant α4β2δ,α6β2δ, and α1β2γ2 $GABA_A$ receptors. GABA concentration–response relations from two-electrode voltage-clamp electrophysiology experiments performed with, or without, 1 or 10 μM DS2 for (A) α4β2δ, (B) α6β2δ and (C) α1β2γ2 receptors expressed in *X. laevis* oocytes. The responses are normalized to the response produced by 30 μM GABA. GABA EC_{50} values calculated from the curves using the Hill equation are α4β2δ [1.5 ± 0.1 μM (n = 8)], α4β2δ + 1 μM DS2 (0.8 ± 0.1 μM, n = 3), α6β2δ (1.3 ± 0.6 μM, n = 7), α6β2δ + 1 μM DS2 (0.5 ± 0.2 μM, n = 3), α1β2γ2 (1.9 ± 0.5 μM, n = 11) and α1β2γ2 + 10 μM DS2 (1.1 ± 0.3 μM, n = 11).

and α6β2δ receptors respectively. By contrast, the maximal enhancement produced by DS2 of the GABA-evoked response mediated by α1β2δ receptors was modest, with a magnitude of enhancement (~2-fold) similar to that found for DS2 modulation of γ2-containing receptors (Figure 1B). Furthermore, the EC_{50} value of DS2 was higher when an α1-subunit rather than an α4- or an α6-subunit was combined with the δ-subunit (see summary EC_{50} values at bottom of Figure 1B).

It is possible that the δ-subunit was not expressed and incorporated into the receptor complex when co-expressed with α1- and β2-subunits. However, DS2 was effective on the same batch of oocytes injected with equivalent cRNAs for α4β2δ and α6β2δ receptors. Furthermore, we found that the GABA-modulatory effect of DS2 acting at α1β2δ receptors was similar to that for oocytes injected with an increased ratio (two- or fivefold) of cRNA encoding for the δ-subunit *cf.* the α4- or β2-subunits (data not shown). Moreover, DS2 did not modulate the effect of GABA at α1β2 receptors (data not shown).

In addressing the influence of the β-subunit on the modulatory actions of DS2, we found no difference in the efficacy or EC_{50} value of DS2 at α4β1δ, α4β2δ and α4β3δ receptors (see summary EC_{50} values at bottom of Figure 1B).

Thus, we conclude from the above, that DS2 is selective for α4βxδ and α6β2δ receptors, exhibiting both a greater efficacy (E_{MAX}) and a more potent modulation (EC_{50}) for α4β1δ, α4β2δ, α4β3δ and α6β2δ receptors *cf.* γ2-containing receptors and the α1β2δ receptor. However, a 10 μM concentration of DS2 did enhance the function of all $GABA_A$ receptorstested, but with limited potency and efficacy.

Concentration–response curves for DS2 were also constructed at GABA I_{max} for the α4β2δ and α1β2γ2 receptor. The modulatory effect of DS2 on the α4β2δ receptor largely exceeds GABA I_{max} for this receptor in the tested concentration range. This is in contrast to the α1β2γ2 receptor where 30 μM DS2 only engenders a 30% current relative to GABA I_{max} (see Figure 1C).

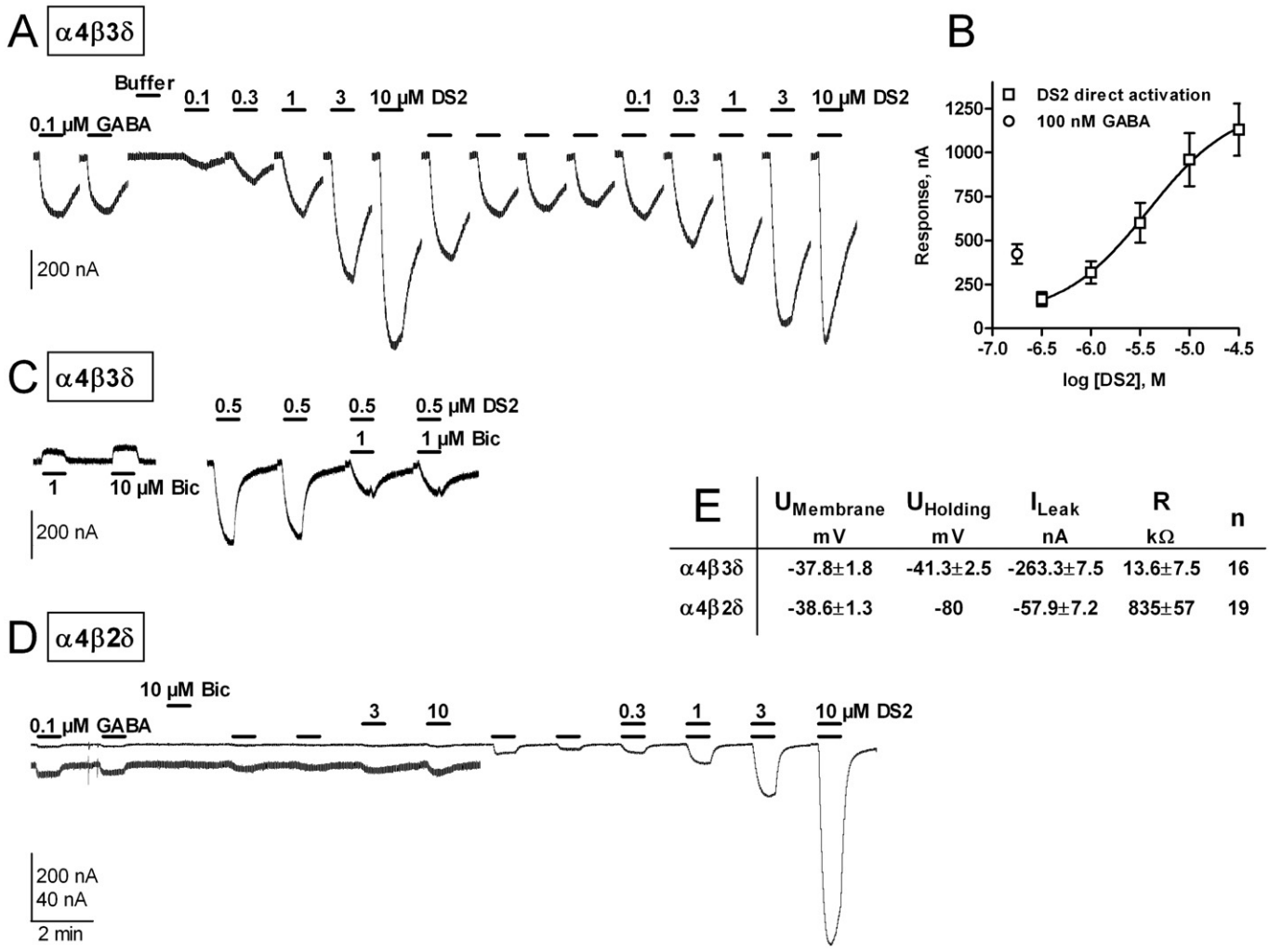
Mechanism of action at $\alpha_4\beta_2\delta$ and $\alpha_6\beta_2\delta$ receptors: DS2 primarily enhances the maximum GABA response with only a modest impact on GABA potency

To investigate in more detail the mechanism of action of this δ-selective ligand, GABA concentration–response relationships were determined in the absence and presence of DS2 for α4β2δ, α6β2δ and α1β2γ2 receptors. For both δ-containing receptors DS2 (1 μM) produced only a modest leftward shift of the GABA EC_{50} (control α4β2δ = 1.5 ± 0.1 μM, n = 8; DS2 = 0.8 ± 0.1 μM, n = 3; control α6β2δ = 1.3 ± 0.6 μM, n = 7; DS2 = 0.5 ± 0.2 μM, n = 3), which was significant (Student's t-test on pEC_{50}) only for the α4β2δ receptor. However, for both α4β2δ and α6β2δ receptors, DS2 produced a large increase of the apparent maximal effect of GABA (see Figure 2A and B). By contrast, a 10 times greater concentration of DS2 did not affect the GABA EC_{50} or the maximal effect at α1β2γ2 receptors (Figure 2C).

Direct receptor activation by DS2?

Previously, it was reported that a close analogue of DS2, namely DS1, had both GABA modulatory effects and direct activating properties (i.e. 'GABA mimetic') at α4β3δ receptors over the same concentration range (3–100 nM) (Wafford *et al.*, 2009). However, in contrast to the earlier report (Wafford *et al.*, 2009), in the absence of GABA, the application of DS2 to *X. laevis* oocytes expressing α4β3δ receptors induced a concentration-dependent (0.3–30 μM) inward current with a calculated EC_{50} of 4.9 ± 2.1 μM, n = 4, and an E_{MAX} of ~2- to 3-fold of that produced by 100 nM GABA as seen in Figure 3A and B.

However, one caveat that needed to be considered before concluding that DS2 exhibits both modulatory and direct activating effects at α4β3δ receptors was that *X. laevis* oocytes expressing α4β3δ receptors exhibited a greater leak current than those expressing most other $GABA_A$ receptors, (e.g. γ2-containing and α4β2δ receptors). The membrane resist-



E

	$U_{Membrane}$ mV	$U_{Holding}$ mV	I_{Leak} nA	R kΩ	n
α4β3δ	-37.8±1.8	-41.3±2.5	-263.3±7.5	13.6±7.5	16
α4β2δ	-38.6±1.3	-80	-57.9±7.2	835±57	19

Figure 3

A comparison of direct receptor activation and positive allosteric modulation by DS2 on human recombinant α4β3δ and α4β2δ receptors. (A) Current traces from two-electrode voltage-clamp electrophysiology experiments addressing the apparent direct activation (traces 4–8) of α4β3δ receptors expressed in *X. laevis* oocytes by DS2. *cf.* the positive allosteric effect of DS2 (traces 13–17). Bars indicate where GABA and/or DS2 are applied for 1 min. Note that the traces are truncated and staggered, omitting the long wash periods – 90 s at lowest concentration, and up to 4 min at the highest concentration of DS2 – to focus on key experimental data only. A new response was elicited first on return to baseline. (B) Concentration–response curves for DS2 recorded at α4β3δ expressing oocytes. Direct activation by DS2 is plotted along with the response to 100 nM GABA. EC_{50} values using the Hill equation were determined to be 4.9 ± 2.1 μM ($n = 4$) for direct activation. (C) Current traces showing the blocking effect of bicuculline on α4β3δ receptors both on leak current (traces 1–2) and on the current elicited by 0.5 μM DS2 without any GABA present (traces 3–6). As in panel A above, the traces are truncated and staggered, omitting the long wash periods to focus on key experimental data only, with a new response being elicited first on return to baseline. (D) Current traces (*truncated and staggered, see above*) from α4β2δ receptors expressed in *X. laevis* oocytes exposed to GABA (traces 1–2), bicuculline (trace 3), DS2 alone (3 and 10 μM, traces 6–7) and DS2 co-applied with GABA (DS2 concentration range, 0.3–10 μM, traces 10–13). The lower traces are amplified to better illustrate the relatively small current responses. (E) Membrane potential and leak current measured from two-electrode voltage-clamp recordings from α4β3δ and α4β2δ receptors expressed in *X. laevis* oocytes. The membrane resistance is calculated as $((U_{holding} - U_{Membrane})/I_{Leak})$.

ance of the *X. laevis* oocyte was calculated by dividing the difference between $U_{holding}$ and $U_{Membrane}$ with I_{Leak}, which revealed a significantly lower resistance of 13.6 ± 2.2 kΩ for oocytes expressing α4β3δ receptors compared with those expressing α4β2δ receptors (835 ± 57 kΩ) as listed in Figure 3E. This leak current could be due to spontaneous openings of the channels in the absence of agonist as reported for some other receptor combinations, e.g. α1βε (Maksay *et al.*, 2003). Two experiments were performed to investigate whether DS2 activates the α4β3δ receptor directly, or whether this inward current is produced by a modulation of spontaneous channel openings. First, we demonstrated that DS2 was not able to elicit direct receptor activation in the absence of GABA when applied to *X. laevis* oocytes expressing α4β2δ receptors, which display little leak current (Figure 3D). Second, bicuculline, an inverse agonist at the orthosteric site (Ueno *et al.*, 1997), was able to inhibit the leak current in *X. laevis* oocytes expressing α4β3δ receptors in a

concentration-dependent manner, whereas it had no effect on *X. laevis* oocytes expressing α4β2δ receptors (compare Figure 3D and C). Moreover, direct activation of the α4β3δ receptor by DS2, in the absence of GABA, was also inhibited by bicuculline (Figure 3C). These observations suggest that α4β3δ receptors demonstrate a degree of spontaneous activity, and that DS2 can positively modulate this activity. Although β3 homomeric receptors do form spontaneously active channels when expressed in *X. laevis* oocytes (Wooltorton *et al.*, 1997), these 'receptors' are not affected by application of DS2 (data not shown), suggesting that the presence of a δ-subunit is required.

As previous studies (Hadley and Amin, 2007) have demonstrated that spontaneous $GABA_AR$ channel opening in oocytes is more prevalent at high levels of receptor expression, we wanted to ensure that our findings above were not an artefact of the oocyte expression system. Therefore, we ran similar experiments (data not shown) to those described above with DS2 in HEK-293 cells (both a stable cell line and in transient transfections). In a HEK-293 stable cell line expressing α4β3δ receptors, we also demonstrated direct activation with DS2, which was blocked by bicuculline. Furthermore, the leak current in these HEK-293 cells stably expressing α4β3δ receptors could be blocked by picrotoxin. When we tested the effects on DS2 on HEK-293 cells engineered to transiently express α4β3δ and α4β2δ receptors, we observed direct activation when the β3, but not when the β2 subunit was present in the receptor construct. These equivalent data sets in HEK-293 and *X. laevis* oocyte expression systems lead us to conclude that it is likely that the presence of a β3 subunit in the receptor construct results in receptors with some constitutive/spontaneous activity, which in turn leads to an apparent 'direct' effect of DS2 at the α4β3δ receptor isoform.

In conclusion, these studies suggest that DS2 does not directly activate GABA α4β2δ or α4β3δ receptors but instead modulates the spontaneous current present when α4β3δ receptors are expressed in *X. laevis* oocytes or HEK-293 cells; a spontaneous activity that does not appear to be evident in mouse L(tk^-) cells expressing α4β3δ (Wafford *et al.*, 2009).

Interaction of DS2 with known sites on the $GABA_A$ receptor

Orthosteric site. At concentrations up to 10 μM, DS2 did not displace [^{3}H]muscimol (2 nM) bound to the orthosteric site of α4β3δ receptors expressed in HEK-293 cells (data not shown). The lack of effect of DS2 on muscimol binding suggests that it does not directly interact with the orthosteric GABA binding site of $GABA_A$ receptors.

Benzodiazepine site. A well-characterized binding site on $GABA_A$ receptors is the benzodiazepine site located on the extracellular interface between α- and γ-subunits (Sigel, 2002; Berezhnoy *et al.*, 2004). To ascertain if DS2 interacted with the benzodiazepine site, we determined if DS2 displaced (i) [^{3}H]flumazenil binding to rat cortical tissue (reflecting binding predominantly to α1β2γ2, but including also α2, α3 and α5 receptor subtypes) and (ii) [^{3}H]Ro 15–4513 binding to α4β3γ2 receptors expressed in HEK-293 cells. In rat cortex, a relatively high concentration of DS2 (30 μM) displaced 30% of specifically bound [^{3}H]flumazenil, suggesting a possible low affinity interaction with the benzodiazepine site or, more likely, that the interaction of DS2 with the receptor causes an allosteric change in the benzodiazepine binding site leading to a change in bound [^{3}H]flumazenil. Nonetheless, we followed up with functional electrophysiology studies and demonstrated that the modest positive modulation of α1β2γ2 receptors by DS2 was not blocked by the benzodiazepine antagonist flumazenil up to a concentration of 100 μM (data not shown). Moreover, DS2 (up to 10 μM) had no effect on the specific binding of [^{3}H]Ro 15–4513 to recombinant α4β3γ2 receptors (data not shown). These experiments indicate that DS2 does not interact with a benzodiazepine-like site at γ-containing $GABA_A$ receptors.

There has been debate as to whether an equivalent site similar to the benzodiazepine site is present on δ-containing $GABA_A$ receptors, as Ro 15–4513 binding to δ-containing receptors has been reported (Wallner *et al.*, 2003; Hanchar *et al.*, 2006; Olsen *et al.*, 2007). However, we did not obtain any specific binding of [^{3}H]-Ro 15–4513 to recombinant α4β3δ receptors expressed in HEK-293 cells (data not shown) and therefore cannot verify the existence of such a site, a finding consistent with others (Korpi *et al.*, 2007).

Etomidate site. Positive allosteric modulation of the $GABA_A$ receptor by the general anaesthetics etomidate and propofol is influenced by the nature of an amino acid residue located within the TM2 domain of the β-subunit (Belelli *et al.*, 1997). Substitution of the naturally occurring asparagine 265 residue of the β2- or β3-subunit, for a methionine residue (N265M) abolishes the GABA-enhancing effects of these compounds at γ2-containing $GABA_A$ receptors (Belelli *et al.*, 1997; Jurd *et al.*, 2003). Both etomidate and DS2 enhanced the current induced by 100 nM GABA at α4β3δ receptors (Figure 4). In agreement with previous studies with γ2-containing receptors, we now demonstrate that the β3 N265M mutation abolished the GABA-enhancing effects of etomidate at δ-containing receptors (i.e. α4β3N265Mδ). By contrast, the GABA enhancing effects of DS2 were not influenced by this β3 N265M mutation (Figure 4). Therefore, the modulation induced by DS2 is not mediated *via* an interaction with this putative anaesthetic binding site.

Neurosteroid site. Previous studies have established that the introduction of two mutations in the α-subunit, namely T235W and Q240W, render $GABA_A$ receptors insensitive to positive allosteric modulation by the endogenous neurosteroid, allopregnanolone (Hosie *et al.*, 2006). Similarly, we demonstrate here that the GABA enhancing actions of allopregnanolone (10 μM) were abolished by these mutations when incorporated in to the α4-subunit (i.e. α4T235W, Q240W β3δ). By contrast, the GABA-enhancing action of DS2 (10 μM) were not influenced by these mutations of the α4-subunit (Figure 4; right-hand bars), suggesting that DS2 does not act *via* the neurosteroid site.

Barbiturate site. The molecular determinants of the barbiturate site have not been identified, so an alternative approach was used to explore whether DS2 and pentobarbital interacted *via* a common site. First, DS2 concentration–response curves were generated in the presence of different concentrations of pentobarbital as shown for 10 μM pentobarbital in

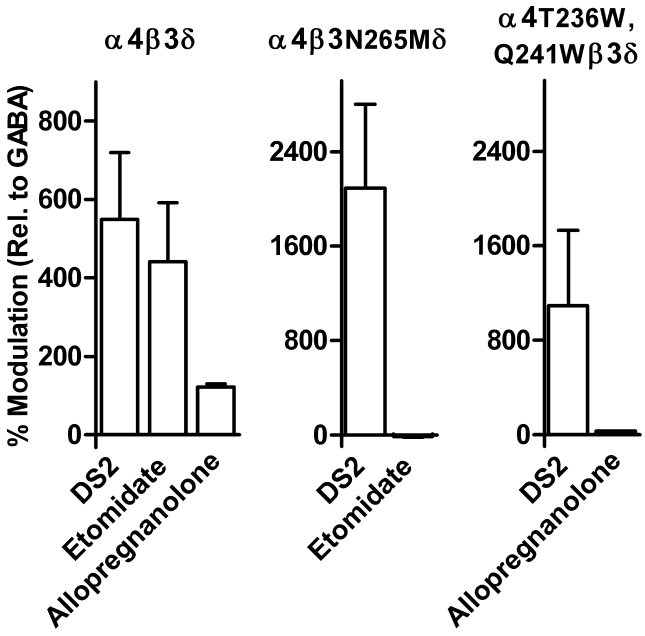


Figure 4

The potential interaction of DS2 with the etomidate or the neurosteroid site of $GABA_A$ receptors. The relative modulation of α4β3δ, α4β3N265Mδ or α4T235W,Q240Wβ3δ receptors expressed in *Xenopus* oocytes by 10 μM DS2, 10 μM etomidate or 10 μM allopregnanolone (see Results section for further details). Percentage (%) modulation was determined relative to a GABA response evoked by 0.1 μM GABA and is shown as an average ± SEM, n = 3–5.

Figure 5A. We found that the DS2 EC_{50} decreased with higher concentrations of pentobarbital, and that clear modulation by DS2 was observed at pentobarbital concentrations up to 100 μM. Thus, pentobarbital does not block the GABA modulatory effect of DS2.

High concentrations of pentobarbital have been reported to directly activate $GABA_A$ receptors (Muroi *et al.*, 2009), but no direct activation with concentrations up to 10 mM pentobarbital were observed for the α4β2δ receptor. However, as seen in Figure 5B, 10 mM pentobarbital gave rise to a rebound current after removal of pentobarbital. This rebound effect was modulated by DS2 with the same potency as for the modulation of GABA (EC_{50} value of 5.3 μM for the modulation of the rebound effect after pentobarbital removal, compared to an EC_{50} value of 5.2 μM for modulation of GABA). As the EC_{50} value for pentobarbital-induced modulation of α4β2δ receptors is between 100 and 300 μM (data not shown), a 10 mM pentobarbital concentration would be expected to saturate the barbiturate modulatory binding site. Because, under these conditions, DS2 can still modulate the receptor, we would suggest that the DS2-induced modulation is not mediated *via* the same site as pentobarbital.

DS2 selectively enhances the function of the extrasynaptic δ-$GABA_A$ receptors of VB thalamocortical neurons: studies using $\delta^{0/0}$ mice

We have previously demonstrated that DS2 produces a concentration-dependent (1–10 μM) enhancement of the

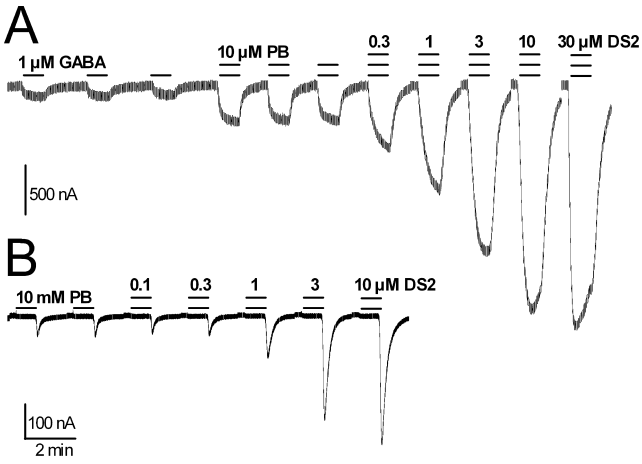


Figure 5

The potential interaction of DS2 with the barbiturate site of $GABA_A$ receptors. (A) Current traces from DS2 concentration–response experiments performed using the co-application of GABA, pentobarbital (PB) and DS2 recorded from *X. laevis* oocytes expressing α4β2δ receptors. Note that the traces are truncated and staggered, omitting the long wash periods – 90 s at lowest concentration, and up to 4 min at the highest concentration of DS2 – to focus on key experimental data only. A new response was elicited first on return to baseline. Traces 1–3 show the response to 1 μM GABA applied alone, traces 4–6 show the response to 10 μM pentobarbital co-applied with 1 μM GABA and traces 7–11 show the modulatory effect of increasing concentrations of DS2 (0.3–10 μM) on responses elicited by 1 μM GABA plus 10 μM pentobarbital. (B) Current traces (*truncated and staggered, see above*) for a DS2 concentration–response experiment on α4β2δ receptors after 10 mM pentobarbital has been washed from the preparation (i.e. modulation by DS2 of a rebound current demonstrable after pentobarbital removal, see text in the Results section for further details).

tonic current of thalamocortical VB neurons (Wafford *et al.*, 2009). To investigate the role of native δ-$GABA_A$ receptors in this effect, we compared VB neurons derived from $\delta^{0/0}$ and wild-type (WT) mice. For neurons derived from WT mice, the $GABA_A$ receptor antagonist bicuculline (30 μM) produced a relatively large outward current (96 ± 8.9 pA, n = 27) (i.e. the 'tonic' current) (Figure 6). The outward current induced by bicuculline (30 μM) was greatly reduced (13 ± 4.7 pA, n = 9; P > 0.001; Student's unpaired t-test) for equivalent neurons derived from the $\delta^{0/0}$ mouse (Figure 6), confirming earlier reports that extrasynaptic δ-$GABA_A$ receptors are important contributors to the tonic current of VB neurons (Porcello *et al.*, 2003; Belelli *et al.*, 2005; Cope *et al.*, 2005; Jia *et al.*, 2005; Chandra *et al.*, 2006). In agreement, with our previous report, the application of 10 μM DS2 to WT VB neurons induced a relatively large inward current (107 ± 9.6 pA, n = 9 – see Figure 6). The effect DS2 on the holding current (11 ± 6.6 pA, n = 5) was greatly reduced (P < 0.001 vs. WT; Student's unpaired t-test) for $\delta^{0/0}$ VB neurons (Figure 6). Concentrations of DS2 that greatly enhance the tonic current of VB neurons have little or no effect on the miniature inhibitory postsynaptic currents (mIPSCs) mediated by α1β2γ2 $GABA_A$ receptors located at the synapse (Wafford *et al.*, 2009). Here, the properties of the mIPSCs recorded from $\delta^{0/0}$ VB neurons were identical to those of WT neurons, confirming earlier reports

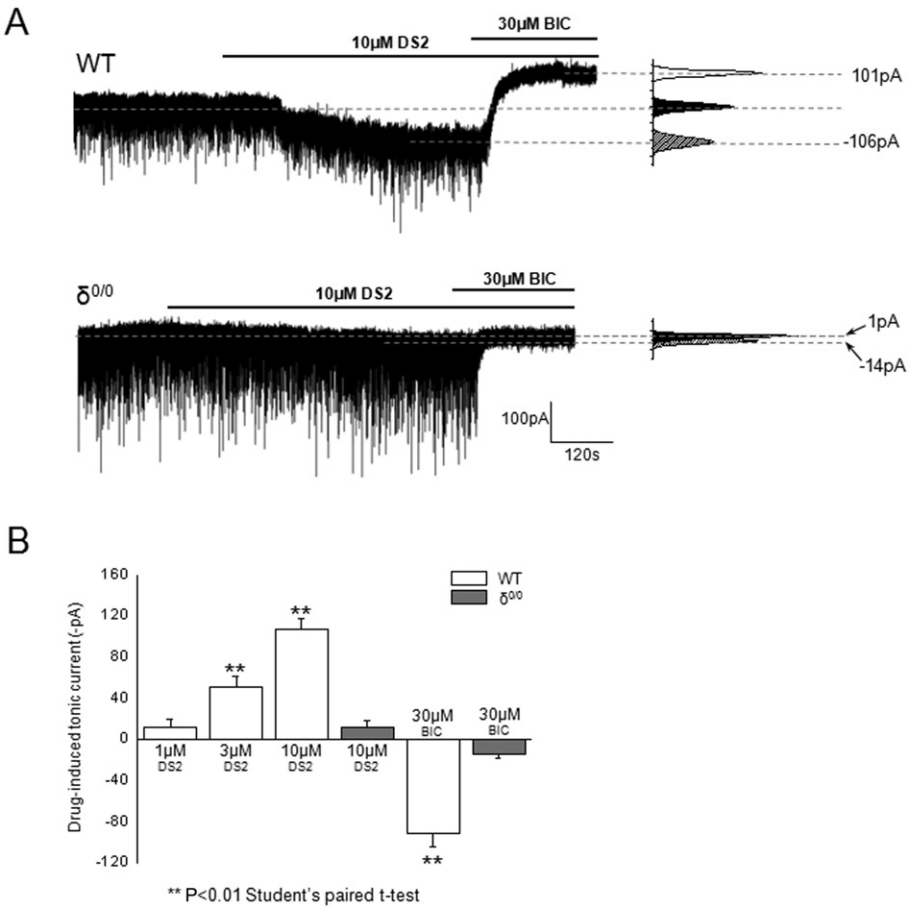


Figure 6

DS2 selectively interacts with extrasynaptic δ-$GABA_A$ receptors of mouse thalamic relay neurons. (A) Top trace: The tonic current (calculated as the difference between the holding current [pA] in the presence and absence of bicuculline 30 μM) recorded from a typical wild-type (WT) mouse thalamic ventrobasal neuron is greatly enhanced by DS2 (10 μM). By contrast, both the effect of DS2 (10 μM) to produce an inward current and the effect of bicuculline (30 μM) to induce an outward current are absent in a typical recording made from mouse thalamic ventrobasal neurons derived from a $\delta^{0/0}$ mouse. To the right of each trace is the corresponding all points histograms of the tonic current for the illustrated recordings under control conditions, in the presence of 10 μM DS2 and 30 μM bicuculline. (B) A bar graph summarizing the inward tonic current induced by 1, 3 and 10 μM DS2 for WT neurons and for 10 μM DS2 for $\delta^{0/0}$ neurons. The trace illustrating the effect of DS2 in WT ventrobasal neurons (panel A) and data for 1 and 3 μM DS2 (panel B) are reproduced from (Wafford *et al.*, 2009). Note that the large outward current induced by bicuculline (30 μM) for WT neurons is absent for equivalent $\delta^{0/0}$ neurons. Data illustrate the mean ± SEM and were obtained from three to seven neurons (**$P < 0.01$, Student's paired *t*-test).

that δ-$GABA_A$ receptors make little contribution to such quantal synaptic events. Furthermore, in agreement with our earlier study on WT VB neurons, DS2 (10 μM) had no effect on the amplitude, kinetics or frequency of $\delta^{0/0}$ mIPSCs (Table 1). In conclusion, these results demonstrate DS2 enhances the tonic conductance mediated by extrasynaptic δ-$GABA_A$ receptors of thalamocortical VB neurons, with no effect on the phasic conductance mediated by synaptic $GABA_A$ receptors; that is, the δ-subunit specificity of this compound exhibited by recombinant receptors is replicated for native neuronal receptors.

Table 1

Effects of 10 μM DS2 on mIPSCs recorded from $\delta^{0/0}$ VB neurones

	Control (n = 5)	10 μM DS2 (n = 5)
Peak amplitude (pA)	–82 ± 5	–85 ± 4
Rise time (ms)	0.5 ± 0.03	0.5 ± 0.02
Area (pC)	–254 ± 26	–285 ± 28
T_{50} (ms)	1.8 ± 0.1	1.9 ± 0.1
T_{90} (ms)	4.9 ± 0.2	5.3 ± 0.3
τ_w (ms)	2.6 ± 0.1	2.8 ± 0.1
Frequency (Hz)	17 ± 2	17 ± 3

Discussion and conclusions

A growing corpus of literature has highlighted the therapeutic potential of drugs that selectively target $GABA_A$ receptor

isoforms that incorporate the δ-subunit. Specifically, targeting α4β2/3δ receptors for treating various CNS (see Introduction and references therein), as well as non-CNS (e.g. type 1 diabetes; see (Mendu *et al.*, 2011) disorders is gaining momentum. It is now evident that δ-$GABA_A$ receptors exhibit a restricted neuronal distribution, are primarily found peri- or extrasynaptically, exhibit distinct biophysical properties compared with synaptic $GABA_A$ receptors and are not affected by clinically used benzodiazepines. Furthermore, drugs that do affect δ-$GABA_A$ receptors are relatively non-selective, exhibiting effects on their synaptic counterparts (e.g. volatile anaesthetics, barbiturates, propofol, etomidate and neurosteroids) (see Introduction and references therein).

In the current study, we have thoroughly characterized the reported α4β3δ-selective PAM DS2 first described by Wafford *et al.* (2009). Although our understanding of the physiological role of these receptors has advanced, given the limited availability of pharmacological tools that target δ-$GABA_A$ receptors, the following discussion compares, where applicable, the profile of DS2 to the only well-described δ-$GABA_A$ receptor selective tool gaboxadol. In addition, two other putative δ-$GABA_A$ receptor-preferring compounds have recently been described, namely the trimethylbenzylamino compound AA29504 (Hoestgaard-Jensen *et al.*, 2010) and a dihydropyrimidinone compound JM-II-43A (Lewis *et al.*, 2010). Therefore, where relevant data are available on these molecules, comparisons are made with our data on DS2.

$GABA_A$ receptor subtype selectivity

We have verified that DS2 is a PAM at α4β3δ receptors but have additionally demonstrated that (i) DS2 is equally effective (i.e. ~ equal efficacy and potency) at α4βxδ and α6β2δ receptors, but not at α1β2δ receptors; (ii) DS2 clearly exhibits selectivity for α4βxδ and α6β2δ receptors *c.f.* $GABA_A$ receptors where a γ-subunit occurs in place of a δ-subunit, whether these receptors *in vivo* occur synaptically (α1β2γ, α2β2γ, α3β2γ) or extrasynaptically (α5β32γ); and (iii) the β-subunit isoform does not influence the PAM activity of DS2 when combined with α4- and δ-subunits. These studies clearly indicate that modulation of $GABA_A$ receptors by DS2 is strongly influenced by whether the δ- or the γ2-subunit is present in the receptor complex and, contrary to gaboxadol, DS2 does not affect α4β3 receptors (Shu *et al.*, 2011). However, our data indicate further subtleties in the determinants of the DS2 selectivity profile not reported previously. First, if a γ2-subunit is present in the receptor complex, the nature of the α-subunit does not influence the limited efficacy, or potency of DS2. By contrast, when investigating δ-$GABA_A$ receptors, it is evident that DS2 modulates α4- and α6- more effectively than α1-containing receptors. These results demonstrate an interesting selectivity profile for DS2 between known *in-vivo* occurring populations of δ-$GABA_A$ receptors.

The best characterized δ-preferring compound described to date is the orthosteric site agonist gaboxadol. Besides the fundamental distinction that gaboxadol is an agonist, whereas DS2 is a PAM, there are also other differences between these two compounds. At γ-receptors (α1–6β1–3γ2), the maximal efficacy of gaboxadol is between 40% and 80% relative to GABA, with EC_{50} of ~100 μM, indicating that relative to GABA, gaboxadol is a relatively low potency agonist of these receptors (Ebert *et al.*, 1994). By contrast, at α6β3δ-, α4β3δ- and α4β3-receptors, gaboxadol has much higher potency (EC_{50} ~10 μM for α6β3δ- and ~50 μM for α4β3δ- and α4β3-receptors), with an apparent maximal efficacy of ~300%, ~200% and 164%, respectively, relative to GABA (Storustovu and Ebert, 2006). Thus, the overall selectivity profile of gaboxadol is α6β3δ > α4β3δ = α4β3 >> αxβ3γ2-receptors. By contrast, DS2 positively modulates α4/6βxδ- but not α4β3γ2-receptors, nor other γ2-receptor populations, and induces no modulation whatsoever of α4β3-receptors. That DS2 is inert at α4β3-receptors makes this drug a very useful tool to confirm the incorporation of a δ-subunit into the receptor complex; that is, the activity of DS2 is fundamentally dependent on the presence of a δ-subunit. Thus, overall, DS2 has a different selectivity profile compared with gaboxadol: α4/6βxδ > α1βxδ >> γ2-receptors > α4β3. Given that many of these receptor populations may exist *in vivo* (McKernan and Whiting, 1996; Pirker *et al.*, 2000; Mortensen and Smart, 2006), the distinct selectivity profiles of DS2 and gaboxadol may lead to different effects in complex biological systems.

Although data on AA29504 (Hoestgaard-Jensen *et al.*, 2010) and JM-II-43A (Lewis *et al.*, 2010) are either limited, or the methodologies and techniques differ from those used here, some comparison with the selectivity profile of DS2 is possible. AA29504 was tested on α4β3δ and α1β3γ2s receptors; and, in common with DS2, this compound preferentially modulates α4β3δ over α1β3γ2S receptors (Hoestgaard-Jensen *et al.*, 2010). However, in more detailed studies addressing the mechanism of action of AA29504, the authors showed that it decreased the EC_{50} for GABA acting at both α4β3δ- and α1β3γ2S-receptors. However, AA29504 only enhanced the maximal response to GABA for α4β3δ-receptors, indicating selectivity for the δ-receptor. Similarly, AA29504 increased the potency of gaboxadol acting at both α4β3δ- and α1β3γ2S-receptors. However, distinct from GABA, for α4β3δ-receptors AA29504 did not enhance but reduced the maximal response to gaboxadol. This difference in the effect of AA29504 on the maximal response to GABA and gaboxadol is probably a reflection of these two molecules being partial and full agonists at δ-$GABA_A$ receptors respectively. JM-II-43A was reported to be a δ-$GABA_A$ receptor compound on the basis of its differential influence on α1β2δ- and α1β2γ2L-receptors. However, many of the experiments were performed at 1 mM saturating GABA concentrations, making it difficult to compare the profile of this molecule with DS2. With GABA being a partial agonist at δ-$GABA_A$ receptors, the window for potentially demonstrating modulatory effects at α4β2δ is greater than for α1β2γ2L receptors where GABA induces much greater open probability (Mortensen *et al.*, 2010). A major difference between DS2 and JM-II-43A is the influence of the α-subunit. Whereas DS2 had a preference for δ-$GABA_A$ receptors combined with the α4 or the α6 over the α1-subunit, JM-II-43A exerted a similar enhancement of δ-$GABA_A$ receptors containing an α1-, α4- or α5-subunit and a reduced effect when an α6-subunit occurred in the receptor complex. Moreover, compared with DS2 (EC_{50} = ~5 μM), JM-II-43A is not very potent (EC_{50} value in the 100 μM range at α1β2δ-receptors).

Mechanism of action

Here we demonstrate that at δ-$GABA_A$ receptors, DS2 (i) increases the apparent maximal effect of GABA at α4βxδ and

α6β2δ receptor subtypes, with only a small impact on GABA potency at δ-receptors; (ii) is a PAM of GABA acting at α4βxδ receptor subtypes, but does not directly activate such receptors, in contrast to the close analogue DS1 (Wafford *et al.*, 2009). For α4β2δ-receptors, DS2 enhances the apparent GABA I_{max} by ~10-fold.

One corollary from the mechanism of action studies is that α4β3δ-receptors expressed in oocytes caused much greater leak current than α4β2δ and all other receptors investigated in this study. This spontaneous activity could be enhanced by DS2. This property was not observed with α4β3δ-receptors expressed in mouse L(tk) cells, although spontaneous activity has been reported in oocytes injected with just β3-subunits (Wooltorton *et al.*, 1997).

Site of action

Using radioligand binding to native tissue or recombinant receptors and functional electrophysiological studies with chimeric receptors, we demonstrated that DS2 did not (i) mediate its effects at α4βxδ-receptors via the orthosteric agonist site; (ii) bind to the 'benzodiazepine' site at various γ_2-subunit containing receptors, and by inference any 'benzodiazepine-like' site suggested to be present on α4β3δ-receptors (Hanchar *et al.*, 2006); (iii) modulate α4βxδ-receptors via an interaction with the etomidate site; (iv) modulate α4βxδ-receptors via an interaction with the neurosteroid site. Finally, DS2 was not likely to modulate α4βxδ-receptors *via* a site common with barbiturates based on interaction studies with pentobarbital. The conclusion that DS2 is not modulating δ-$GABA_A$ receptors *via* a site common with barbiturates is based on an indirect methodology compared to our studies exploring the orthosteric, benzodiazepine, etomidate and neurosteroid sites. Nonetheless, collectively, these findings suggest a unique binding site for DS2 with efficacy critically dependent on the presence of the δ-subunit in the $GABA_A$ receptor complex.

Previous studies have reported the specific binding of [^{3}H]-Ro 15–4513 to recombinant α4/6β2/3δ receptors with low nanomolar affinity, suggesting the presence of a 'benzodiazepine-like' site on δ-$GABA_A$ receptors. However, in agreement with others (Korpi *et al.*, 2007), we found no specific binding of [^{3}H]-Ro 15–4513 to α4β3δ-receptors expressed (either transiently or stably) in HEK-293 cells. These HEK-293 cells expressing α4β3δ receptors gave clear signals to GABA in a FLIPR assay *per se* (data not shown) and responded to DS2, indicating the presence of the δ-subunit in the complex. Moreover, if modulation by DS2 of α4βxδ receptors was mediated through its binding to a 'benzodiazepine-like' site on α4βxδ receptors, then some displacement of [^{3}H]-Ro 15–4513 binding to α4β3γ2 expressed in HEK-293 cells, or a greater degree of displacement of [^{3}H]flumazenil binding to cortex might have been observed. Furthermore, there was no influence of flumazenil on the limited modulation of α1β2γ2-receptors by DS2 in electrophysiological studies, indicating no major interaction of DS2 with the known benzodiazepine site at this receptor isoform.

Native neuronal $GABA_A$ receptors

We previously demonstrated that DS2 enhanced a tonic current in mouse thalamocortical VB neurons (Wafford *et al.*, 2009). Here we additionally revealed that this effect was mediated *via* δ-containing receptors as the modulation of the tonic current was not evident in VB neurons derived from the $\delta^{0/0}$ mouse. The predominant δ-$GABA_A$ receptor population in VB neurons is the α4β2δ isoform, which mediates the actions of gaboxadol in these thalamic neurons (Belelli *et al.*, 2005; Chandra *et al.*, 2006; Peden *et al.*, 2008) and potentially the effects of gaboxadol on sleep (Wafford and Ebert, 2006; Winsky-Sommerer *et al.*, 2007). As shown previously for gaboxadol, there was no influence of DS2 on mIPSCs mediated by synaptic α1β2γ2 receptors in VB neurons obtained from WT or $\delta^{0/0}$ mice (Belelli *et al.*, 2005; Chandra *et al.*, 2006; Peden *et al.*, 2008). δ-containing receptors are not only found in the thalamus (Peng *et al.*, 2002) – in some brain regions, the δ-subunit appears to preferentially partner with α1- and α6-subunits (e.g. hippocampal interneurons and cerebellar granule cells, respectively) (Nusser *et al.*, 1998; Sun *et al.*, 2004; Mangan *et al.*, 2005), rather than α4-subunits (e.g. dentate gyrus granule cells and VB thalamocortical neurons) (Chandra *et al.*, 2006). In this respect, it is interesting that DS2 appears to discriminate between δ-receptor populations, in that it selectively modulated δ-$GABA_A$ receptors incorporating an α4- or α6-, rather than a α1-subunit. Future studies exploring the effects of DS2 on neurons where α1β2δ and α6β2δ populations predominate would clarify whether this selectivity at recombinant receptors is observed in native preparations expressing these subtypes.

In vivo *properties*

Although not reported in this paper, we have attempted to ascertain the utility of DS2 as an *in vivo* tool compound. However, in an initial pharmacokinetic study in both mice and rats, we found that whereas plasma exposure after a 10 mg·kg^{-1} p.o. dose of DS2 was very high in both rats (C_{max} ~30 μM) and mice (C_{max} ~15 μM), in both species, DS2 had very poor brain penetration (brain/plasma ~0.1). Further assessment in the rat at a higher dose (30 mg·kg^{-1}, p.o.), as well as being delivered *via* other routes of administration (s.c. and i.p.), did not result in any significant improvement in brain-penetrant properties. This poor brain/plasma profile was confirmed by a lack of effect of DS2 at doses up to 100 mg·kg^{-1} p.o. in animal models of gross behaviour (Irwin screen, locomotor activity, rotarod), anxiety (e.g. zero maze and stress-induced hyperthermia), psychosis (MK-801 hyperactivity, PPI) and pain (formalin). Although it is plausible that these models might be unresponsive to a compound with the $GABA_A$ receptor selectivity profile of DS2 and that more appropriate models should be considered [e.g. (Maguire and Mody, 2007; Smith *et al.*, 2007), given the poor brain/plasma ratio achieved with DS2 in rodents and the clear *in vivo* effects described for gaboxadol in similar models (Wafford and Ebert, 2006; – see Introduction), this explanation is unlikely. Clearly, this does not preclude DS2 being administered directly into brain ventricles or discrete brain regions in future studies. However, potentially of more interest, given the excellent plasma exposure of DS2 in mice and rats, is to consider assessing DS2 in animal models of disorders with a peripheral/non-CNS basis (Mendu *et al.*, 2011).

In contrast to the poor CNS bioavailability of DS2, AA29504 (Hoestgaard-Jensen *et al.*, 2010) over a range of doses has been reported to have an excellent brain/plasma

ratio of ~1.5 in rats with absolute brain concentrations reaching 1.6 μM at a dose of 4 mg·kg^{-1} after s.c. administration. At a dose of 4 mg·kg^{-1}, some *in vivo* effects were reported in rodents: anxiolysis, impairment of rotarod performance and a synergistic interaction with ethanol leading to impaired rotarod performance. Although these data are of interest, as noted above a 1 μM concentration (brain level in rat after 4 mg·kg^{-1} = 1.6 μM) modulates both IPSCs and tonic inhibition (in the presence of gaboxadol) in cortical slices. Furthermore, since AA29504 is an analogue of the neuronal K_v7 channel opener retigabine, care should be exercised in associating its actions exclusively with $GABA_A$ receptors. Indeed, the authors indicate that in the rat amygdaloid kindling model of partial seizures the effects seen with AA29504 at 10 mg·kg^{-1} are likely to be linked to activation of K_v7 channels. Moreover, the effects described at the 4 mg·kg^{-1} dose in the anxiety, rotarod and ethanol interaction tests are consistent with the profile of retigabine (Blackburn-Munro *et al.*, 2005; Korsgaard *et al.*, 2005). Recently, Damgaard *et al.* (2011) have shown that AA29504 reverses recognition memory deficits in an animal model of schizophrenia, albeit retigabine is also effective in schizophrenia models (Sotty *et al.*, 2009).

In conclusion, we have conducted an in-depth characterization of DS2 demonstrating its unique selectivity for a subset of δ-receptors amongst a range of human $GABA_A$ receptors, verified that this selectivity is maintained when assessing native (rodent) $GABA_A$ receptors, shown that the predominant mechanism of action of DS2 was to enhance the maximal GABA response at δ-$GABA_A$ receptors, and determined that DS2 was unlikely to mediate its effects *via* currently known sites on $GABA_A$ receptors. DS2 was distinguished from gaboxadol in having a different selectivity profile, in addition to being a PAM rather than an agonist. Although not a convenient tool after systemic administration for *in vivo* studies of CNS disorders, direct brain administration and assessment of DS2 in animal models of peripheral disorders is feasible. This in-depth characterization of DS2 increases our understanding of effects being mediated by this and other δ-subunit selective drugs and suggests that the effects of DS2 are mediated by an as yet uncharacterized site on δ-$GABA_A$ receptors.

Acknowledgements

We would like to acknowledge Carsten Jessen for the synthesis of DS2 and Elsebeth Østergaard Nielsen for help with the binding experiments. Work was supported by an AJ Clark Studentship (JJL & DB), MRC Grant G1000008 (DB & JJL) and Tenovus Scotland (DB).

Conflict of interest

None.

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