Molecular Determinants underlying Delta Selective Compound 2 (DS2) Activity at δ-Containing GABA_A Receptors


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Running Title Page

a) Running title:
Identification of the DS2 interaction site

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Br-DS2OPh, N-(6-bromo-2-(thiophen-2-yl)imidazo[1,2-a]pyridin-3-yl)-4-phenoxybenzamide; Cryo-EM, cryogenic electron microscopy; DS2, 4-chloro-N-[2-(2-thienyl)imidazo[1,2-a]pyridin-3-yl]benzamide; DPBS, Dulbecco’s phosphate buffered saline; DS2OMe, (4-methoxy-N-[2-(thiopen-2-yl)imidazole[1,2-a]pyridine-3-yl]benbamide); ECD, extracellular domain; FLIPR, fluorometric imaging plate reader; FMP, FLIPR membrane potential; GABA_A, GABA type A receptor; HBSS, Hank’s balanced salt solution, HEK, human embryonic kidney; PAM, positive allosteric modulator; RFU, relative fluorescence units; TMD, transmembrane domain; WT, wildtype.
Abstract

Delta selective compound 2 (DS2) is one of the most widely used tools to study selective actions mediated by δ subunit-containing GABA_A receptors. DS2 was discovered over 10 years ago, but despite great efforts, the precise molecular site of action has remained elusive. Using a combination of computational modeling, site-directed mutagenesis and cell-based pharmacological assays, we probed three potential binding sites for DS2 and analogs at α_4β_1δ receptors: an α_4(+)δ(-) interface site in the extracellular domain (ECD), equivalent to the diazepam binding site in αβ_2γ receptors, and two sites in the transmembrane domain (TMD); one in the α_4(+)/β_1(+) and one in the α_4(-)/β_1(+) interface, with the α_4(-)/β_1(+) site corresponding to the binding site for etomidate and a recently disclosed low-affinity binding site for diazepam. We show that mutations in the ECD site did not abrogate DS2 modulation. However, mutations in the TMD α_4(+)/β_1(-) interface, either α_4(S303L) of the α_4(+)-side or β_1(I289Q) of the β_1(-)-side, convincingly disrupted the positive allosteric modulation by DS2. This was consistently demonstrated both in an assay measuring membrane potential changes and by whole-cell patch-clamp electrophysiology and rationalized by docking studies. Importantly, general sensitivity to modulators was not compromised in the mutated receptors. This study sheds important light on the long-sought molecular recognition site for DS2, refutes the misconception that the selectivity of DS2 for δ-containing receptors is caused by a direct interaction with the δ-subunit, and instead points towards a functional selectivity of DS2 and its analogs via a surprisingly well-conserved binding pocket in the TMD.
Significance Statement:

δ-Containing GABA<sub>δ</sub> receptors represent potential drug targets for the treatment of several neurological conditions with aberrant tonic inhibition, yet, no drugs are currently in clinical use. With the identification of the molecular determinants responsible for positive modulation by the known compound DS2, the ground is laid for design of ligands that selectively target δ-containing GABA<sub>δ</sub> receptor subtypes, for better understanding of tonic inhibition, and, ultimately, for rational development of novel drugs.
Introduction

Inhibition in the brain is primarily mediated by GABA acting through GABA receptors, with the ionotropic GABA type A receptors (GABA\textsubscript{AR}) being responsible for fast inhibition. Thus, GABA\textsubscript{AR}s play an essential role in transmitting inhibitory signaling in the brain. Structurally speaking, GABA\textsubscript{AR}s belong to the Cys-loop receptor family of pentameric receptor complexes and are composed from a repertoire of 19 different subunits in mammals, with the most commonly expressed in the CNS being $\alpha_{1-6}$, $\beta_{1-3}$, $\gamma_{1-3}$ and $\delta$ (Olsen and Sieghart, 2009). The subunit stoichiometry of the archetypical GABA\textsubscript{A} receptor is $2\alpha$, $2\beta$ and a third subunit, most typically a $\gamma$ or a $\delta$-subunit, but other stoichiometries have also been reported (Olsen and Sieghart, 2009). Studies on the subunit arrangement of the most abundantly expressed synaptic subtype, $\alpha_1\beta_2\gamma_2$, and a number of other $\gamma$-containing subtypes, show that the subunits are arranged as $\gamma$-$\beta$-$\alpha$-$\beta$-$\alpha$ in a counterclockwise fashion around the central ion channel (Tretter \textit{et al.}, 1997; Baumann \textit{et al.}, 2002). Although it is generally accepted that the $\delta$-subunit in its cognate receptors simply replaces the $\gamma$-subunit with respect to arrangement (Barrera and Herbert, 2008), this is still not unequivoically established (Baur \textit{et al.}, 2009; Kaur \textit{et al.}, 2009; Wagoner and Czajkowski, 2010; Patel \textit{et al.}, 2014). Irrespective, the orthosteric binding sites are located at the $\beta^{(+)}\alpha^{(-)}$ interfaces in the extracellular domain (ECD), and a number of allosteric binding sites have also been identified in the subunit interfaces of both the ECD and TMD (Olsen, 2018). These include for example the benzodiazepine site in the ECD $\alpha^{(+)}\gamma^{(-)}$ interface, responsible for mediating the anxiolytic and sleep-inducing effect of the benzodiazepines, including diazepam (Valium\textsuperscript{®}), widely used in the clinic (Sigel and Steinmann, 2012; Simeone \textit{et al.}, 2019).

The $\delta$-containing GABA\textsubscript{AR}s are located primarily at extrasynaptic sites where they mediate tonic (persistent) inhibition (Mody, 2001; Farrant and Nusser, 2005), hence controlling neuronal excitability (Belelli \textit{et al.}, 2009). Tonic inhibition is involved in various
physiological responses and pathophysiological conditions (Lee and Maguire, 2014), underlining a continued interest in targeting these receptors in conditions like insomnia (Wafford and Ebert, 2006), ischemic stroke (Clarkson et al., 2010; Lie et al., 2019), some forms of epilepsy (Cope et al., 2009), and peripheral immunomodulation (Yocum et al., 2017; Neumann et al., 2019). However, compared to the synaptic γ-containing receptors, pronounced insight into the physiological and pathophysiological role of δ-containing receptors is still limited by the low number of potent and selective compounds.

One highly used model compound with selectivity for δ-containing receptors is the positive allosteric modulator (PAM) delta selective compound 2 (DS2; 4-chloro-N-[2-(2-thienyl)imidazo[1,2-a]pyridin-3-yl]benzamide) (Wafford et al., 2009). DS2 is extensively used as a tool compound to confirm the presence of δ-receptor-mediated tonic currents both in vitro and in vivo (Wongsamitkul et al., 2016; Falk-Petersen et al., 2017; Zhang et al., 2017; Dalby et al., 2020). DS2 was identified in a screening campaign and reported as a δ-selective PAM at α4β3δ GABAARs, showing no or limited effects at α4β3γ2 and α1β3γ2 receptors (Wafford et al., 2009). This selectivity was confirmed in thalamic relay neurons, where only extrasynaptic tonic currents were enhanced (Wafford et al., 2009) and using δ−/− mice (Jensen et al., 2013). DS2 displays limited brain permeability (Jensen et al., 2013) but was, nonetheless, shown to improve recovery after stroke in mice, plausibly by dampening peripheral immune activation (Neumann et al., 2019). Recently, a methoxy analog of DS2, termed DS2OMe, was identified and confirmed to have potential as a positron emission tomography tracer for visualization of δ-containing receptors in brains of larger mammals such as pig (L’Estrade et al., 2019).

In 2018, the first cryogenic electron microscopy (cryo-EM) structure of a human GABAAR pentamer α1β2γ2 was published (Zhu et al., 2018). Following, a structure of the α1β3γ2 receptor was solved in complex with diazepam, revealing both the known high-affinity
diazepam binding site in the α(+)γ(−) interface in the ECD, and a novel low affinity binding site located in the α(+)β(−) interface of the TMD (Masiulis et al., 2019).

Based on the notion that binding pockets evolved through nature are often highly conserved, combined with the structural similarities between DS2 and the benzodiazepine site ligand zolpidem (Rostrup et al., 2020), we hypothesized that similar pockets are present in δ-containing subtypes and that either of them could represent the long-sought-after DS2 site. We here report the identification of two residues, α4(S303) and β1(I289) within the predicted α4(+)β1(−) TMD interface of α4β1δ receptors as necessary for DS2 modulation. These findings are supported by docking of DS2 analogs into the identified binding pocket.

Materials and Methods

General

The study is exploratory by nature and follows the guidelines detailed (Michel et al., 2020). Data collection were in some cases defined by some preset standards as detailed under each experimental section.

Chemicals and materials

The compounds DS2; (4-chloro-N-[2-(2-thienyl)imidazo[1,2-a]pyridin-3-yl]benzamide), AA29504; ([2-amino-4-(2,4,6-trimethylbenzylamino)-phenyl]-carbamic acid ethyl ester), etomidate; ((R)-1-(1- phenylethyl)-1H-imidazole-5-carboxylic acid ethyl ester), picrotoxin and GABA were obtained from Tocris Bioscience (Bristol, UK). DS2OMe (4-methoxy-N-[2-(thiopen-2-yl)imidazole[1,2-a]pyridine-3-yl]benzamide) was synthesized in-house as described previously (Yakoub et al., 2018), purity test by HPLC and combustion analysis: calcd for C_{19}H_{15}N_{3}O_{2}S: 350.09, found 350.09. Reverse phase-HPLC: R_t (MeCN/H_2O, 1:1) =
6.75 min, purity >99%. DMEM with GlutaMAX-I, FBS, penicillin-streptomycin, hygromycin B, trypsin-EDTA, DPBS and HBSS were purchased from Life Technologies (Paisley, UK). DMSO, HEPES, MgCl₂, CaCl₂, poly-D-lysine and MgATP were purchased from Sigma-Aldrich (St. Louis, MO, USA). The fluorometric imaging plate reader (FLIPR) membrane potential (FMP) Blue dye was purchased from Molecular Devices (Crawley, UK) and Polyfect transfection reagent from Qiagen (West Sussex, UK). Stocks of DS2 and DS2OMe were prepared at 1 mM and 10 mM concentrations in DMSO with final DMSO concentration <0.1%. Due to moderate solubility and 4x concentrations used in the FMP assay, the buffer was preheated to 37 °C in a water bath before addition of compound and preparation of serial dilutions. Only stocks with final concentrations below 12 μM were used for further dilutions. Furthermore, higher concentrations were prepared separately.

**Cells and transfections**

A human embryonic kidney (HEK)293 Flp-In cell line stably expressing the human δ-GABA₆R subunit (Falk-Petersen *et al.*, 2017) was used for transfection with human α- and β-subunits to express recombinant wildtype (WT) and mutant GABA₆Rs and transfection ratios optimized as described (Falk-Petersen *et al.*, 2017). Cells were maintained in DMEM containing GlutaMAX-I, supplemented with 10% FBS and 1% penicillin-streptomycin and kept in an incubator at 37 °C and a humidity of 5% CO₂. 200 μL/ml hygromycin B was added to the media as positive selection. Transfection was performed using Polyfect (Qiagen) following the manufacturer’s instructions except for using half the volume of transfection reagent for each transfection. α- and β-subunits were co-transfected in a 1:1 ratio for FMP experiments, and for patch-clamp experiments additionally co-transfected with GFP in a 0.5:1:1 ratio (0.8 μg:1.6 μg:1.6 μg in 6 cm culture dishes) in order to visualize transfected cells.
Plasmids and mutant constructs

The plasmids used for transfection to transiently express GABA<sub>A</sub> receptors have been described previously (Falk-Petersen et al., 2017). The WT human α<sub>4</sub> and β<sub>1</sub> subunits were subcloned into the pUNIV vector (Addgene, Cambridge, MA, USA) and the human δ-subunit into the pcDNA5/FRT vector (Invitrogen, Paisley, UK) using the δ-construct described previously (Falk-Petersen et al., 2017). Plasmids carrying single and double mutations were generated and sequence-verified by GenScript (Piscataway, NJ, USA). The numbering of the mutants refers to the sequences with the signal peptide included.

Generation of stable cell lines

Mutations introduced into the δ-subunit were established as stable HEK293 Flp-In<sup>TM</sup> cell lines (Invitrogen), generating a stable cell line for each mutant. The stable cell lines were generated using the pcDNA/FRT/V5-His TOPO TA Expression kit (Invitrogen) performed according to the manufacturer’s protocol and as described previously (Falk-Petersen et al., 2017), except for using 25 μL Polyfect and 4 μg DNA for transfection in a 10 cm culture dish.

FLIPR membrane potential (FMP) assay

The FMP assay was performed exactly as described previously (Falk-Petersen et al., 2017). In brief, 48 hours before the assay, cells were transfected. 16-20 hours later, cells were plated into clear-bottomed PDL (poly-D-lysine)-coated black 96-well plates in a number of 50,000 cells/well, suspended in cell media, and placed in an incubator at 37 °C with a humidity of 5% CO<sub>2</sub> until performing the assay. 44-48 hours post-transfection, the media was removed, cells were washed in assay buffer (100 μL/well) and incubated in 100 μL/well 0.5 mg/mL
FMP blue dye freshly dissolved in assay buffer (HBSS containing 20 mM HEPES adjusted to pH 7.4 and supplemented with 2 mM CaCl$_2$ and 0.5 mM MgCl$_2$) for 30 min shielded from light in an incubator at 37° C and a humidity of 5% CO$_2$. Ligand solutions were prepared in 4x assay buffer and added to a ligand plate, which was placed in the FLEXstation3 plate reader (Molecular Devices, Crawley, UK), preheated to 37° C for temperature equilibration for 10-15 min. After transferring the cell plate to the reader, the fluorescence was measured at baseline and after ligand addition by detecting emission at 560 nm caused by excitation at 530 nm.

**FMP experimental design and data analysis**

For FMP experiments, some preset formats were used for assay design and data analysis. Compound-induced signals were reported as changes in relative fluorescence units (ΔRFU), with the signal given as the difference between the average of the baseline signal (approx. 30 s recording) subtracted the peak response (or minimum response for decreases in baseline). All raw traces were manually inspected for obvious artefacts after compound addition. For high concentrations of DS2 (1-20 µM), we regularly observed negative RFU values below the buffer responses that in certain cases were excluded (see below). This phenomenon was independent of receptor subtype as it was observed for both δ-HEK and mock cells. The phenomenon was less pronounced for DS2OMe why this compound was preferred in some sub-studies. To circumvent this problem, we set up the following exclusion criteria: negative ΔRFU or decreased ΔRFU values for high concentration (>1 µM) of DS2 and DS2OMe compared to the ΔRFU for a lower concentration in the same experiment (indicative of precipitation). Additionally, curve fittings resulting in ambiguous EC$_{50}$ values, and R$^2$ values lower than 0.80, due to very small responses, were omitted from analyses resulting in the following number of excluded experiment (excluded/total number) using either DS2 or
DS2OMe at the given receptor subtypes: \(\alpha_4\beta_1\delta\), 6/18; \(\alpha_4(F133A)\), 2/7; \(\alpha_4(F133L)\), 4/7; \(\alpha_4(R135A)\), 6/10; \(\alpha_4(R135H)\), 4/7; \(\alpha_4(G191A)\), 4/8; \(\alpha_4(G191E)\), 2/7; \(\alpha_4(G191L)\), 4/7; \(\delta(E71L)\), 0/5; \(\delta(F90A)\), 0/4; \(\delta(H204A)\), 1/5; \(\delta(S155Q)\), 1/5; \(\delta(A73N)\), 1/5; \(\alpha_4(S303L)\), 0/3; \(\alpha_4(L302Y)\), 1/5; \(\alpha_4(L302Y,S303L)\), 2/5; \(\beta_1(I289Q)\), 1/8; \(\beta_1(S290F)\), 0/4. For mutants, experiments were generally performed in 3-5 independent experiments with technical triplicates, which was decided prior to execution, based on the level of variation observed in previous work. For technical reasons, a few experiments had to be conducted at \(n=6-7\) (Table 1, Table S5). WT data was performed in 8-11 independent experiments as they served as controls across experiments.

Experimental data are shown in scatter plots with 95% confidence intervals with \(n’s\) given in the figure legends. Curves were normalized to GABA to allow side-by-side representation and depicted as representative data (means ± S.D.). Mean EC\(_{50}\) values and pEC\(_{50}\) values with 95% confidence intervals are collected in tables along with statistical values. Concentration-response curves were fitted using nonlinear regression, with log-transformed concentrations as x-values, using the four-parameter concentration-response equation:

\[
\text{Response} = \text{bottom} + \frac{\text{top} - \text{bottom}}{1+10^{([\log \text{EC}_{50} - x]n_H)}}
\]

to determine the EC\(_{50}\) value and Hill slope (\(n_H\)). The ‘bottom’ and ‘top’ denotes the upper and lower non-constrained plateau of the curve, respectively. The calculated EC\(_{50}\) values were log-transformed to obtain mean pEC\(_{50}\) values. Statistical analysis of mutated receptors was performed on the pEC\(_{50}\) values using the two-sided Welch’s \(t\)-test compared to WT, correcting for multiple comparison using the original FDR method of Benjamini and Hochberg with a discovery rate of 0.05. Both adjusted and un-adjusted P-values are reported. Data analysis and statistics were performed in GraphPad Prism (v. 8.4.3; GraphPad, San Diego, CA, USA).
Whole-cell patch-clamp electrophysiology

Whole-cell patch-clamp experiments were performed on δ-HEK cells transiently co-expressing WT or mutant α- and β-subunits and GFP as described previously (Falk-Petersen et al., 2020). In short, the transfected cells were transferred to 35 mm petri dishes (100,000-200,000 cells) the day prior to performing the experiment. On the day of experiment, cell media was exchanged for ABSS (containing the following (in mM): NaCl 140, KCl 3.5, Na₂HPO₄ 1.25, MgSO₄ 2, CaCl₂ 2, glucose 10, and HEPES 10; pH 7.35) at room temperature (20-24 °C), before placing at the stage of an Axiovert 10 microscope (Zeiss, Germany). Viewing the cells at 200× magnification and visualizing cells containing green fluorescent protein with UV light from an HBO 50 lamp (Zeiss, Germany), the cells were approached with micropipettes of 1.2–3.3 MΩ resistance manufactured from 1.5 mm OD glass (World Precision Instruments, Sarasota, Florida, USA) on a microelectrode puller, model PP-830 (Narishige, Tokyo, Japan). The micropipettes contained an intracellular solution composed of the following (in mM): KCl 140, MgCl₂ 1, CaCl₂ 1, EGTA 10, MgATP 2, and HEPES 10; pH 7.3.

Recordings were made from cells in the whole-cell configuration using the standard patch-clamp technique in voltage mode and an EPC-9 amplifier (HEKA, Lambrecht, Germany). The clamping potential was -60 mv and series resistance was 80% compensated. Whole-cell currents were recorded using Pulse and PulseFit software (v.8.80, HEKA). Ligand solutions, prepared in ABSS, were applied using two VC3-8xP pressurized application systems feeding into a sixteen-barreled perfusion pipette (ALA Scientific Instruments Inc., Farmingdale, NY, USA) ending approximately 100 μm from the recorded cell. PAMs were tested using co-application with a concentration of GABA corresponding to GABA EC₁₀-₃₅ at the respective receptor subtype. Preapplication was not used for DS2 and DS2OMe, as results from preliminary experiments showed no difference in the size of the peak current with and
without preapplication of the PAM. PAMs and GABA was co-applied for 10-30 s until the peak current was reached. Agonists was applied for 5 s. Between compound applications, compound-free ABSS was applied from one of the barrels in order to quickly remove the compounds from the cell and cells were allowed to recover for 1 min before the next ligand application.

Patch-clamp data analysis and statistics

As for FMP, some preset formats regarding assay design and data analysis were used. All currents were normalized to the maximum GABA current and given as % $\frac{I}{I_{\text{max}}}$. All currents are reported as normalized mean currents with 95% confidence interval. Based on previous experience, currents from at least five different cells from at least two transfections were used. In a few cases, up to 16 cells were used due to technical reasons (see Fig. S4-S5). All n’s are given in the figure legends. Datasets with GABA controls (0.1-0.5 \( \mu \text{M} \)) deviating from GABA EC\(_{10}\) to EC\(_{35}\) were excluded from the analysis.

Statistical analysis was applied to test whether the PAMs potentiated the GABA control response using two-side Welch’s t-test as for FMP data. Analysis of currents was performed using Pulse and Pulsefit (HEKA) and current traces were visualized using IgorPro (v. 6.2.2.2, Wavemetrics, Lake Oswego, OR, USA). Collected data and statistical analysis were performed using GraphPad Prism (v. 8.4.3).

Homology model for the extracellular domain (ECD) binding site

The homology model of the ECD $\alpha_4^{(+)}\beta_1^{(-)}$ interface has been described previously (Rostrup et al., 2020). The model was used to identify residues for the mutational study based on the docking of DS2 into the model described previously (Rostrup et al., 2020).
Homology model for the transmembrane domain (TMD) binding site

The homology model of the transmembrane part of the $\alpha_4\beta_1$ interface was constructed with Modeller 9.24 (Webb and Sali, 2016) using the $\alpha_1\beta_3$ interface from the $\alpha_1\beta_3\gamma_2L$ crystal structure (PDB code 6HUP (Masiulis et al., 2019)) as template. Model and template sequences of the TM helices and the connecting loops making up the subunit interface were obtained and aligned in UniProt (http://www.uniprot.org/ (Consortium, 2018)); sequence IDs $\alpha_4$ P48169, $\beta_1$ P18505, $\alpha_1$ P114867 and $\beta_3$ P28472. To adhere as much as possible to the very closely related template structure, the “very fast” keyword was utilized to output the initial model that is only subjected to a brief optimization, thus, retaining the copied coordinates for all conserved residue positions. This procedure was selected based on the high sequence similarities and assumed structural conservation combined with the fact that the binding site residues are optimized relative to the ligand in following computational steps.

Induced-fit docking of DS2 into the transmembrane $\alpha_4^{(+)}\beta_1^{(-)}$ site and in silico mutagenesis

The homology models were prepared for docking with the Protein Preparation Wizard (Schrödinger Release 2020-2, Schrödinger, LLC, New York, NY, 2020, (Sastry et al., 2013)) using default settings. The chemical structure of DS2 was downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov (Kim et al., 2018) CID: 979718) and the analogs DS2OMe (L’Estrade et al., 2019) and Br-DS2OPh (Rostrup et al., 2020) were built from DS2 in MarvinSketch 20.15.0, ChemAxon (http://www.chemaxon.com). All three ligands were prepared for docking with default settings in LigPrep (Schrödinger Release 2020-2) and used for induced-fit docking in the model of the transmembrane $\alpha_4\beta_1$ interface with the Standard Protocol. The binding site center was defined by Ser303 and Ala324 from $\alpha_4$ plus Pro253 and Ile289 from $\beta_1$. The ligand length was set to $\leq 14$ Å and XP precision was used in the re-docking step, while all other settings were default. The best-scoring docking poses
according to the IFD score were selected for each compound as the most likely binding mode. *In silico* mutagenesis was performed with the built-in protein mutagenesis wizard in PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.) and the backbone dependent rotamer library selecting the most probable rotamer with the fewest steric clashes with surrounding residues. For the $\alpha_4$S303L and $\beta_1$I289Q mutations the first (1 of 4, 47.4%) and second (2 of 16, 14.6%) most likely rotamers were selected, respectively.

**Results**

To identify central residues for the activity of DS2 at $\delta$-containing GABA$_A$Rs, we systematically investigated three potential binding pockets: one in the ECD (the $\alpha_4^{(+)}\delta^{(-)}$ interface), and two in the TMD (the $\alpha_4^{(+)}\beta_1^{(-)}$ and the $\beta_1^{(+)}\alpha_4^{(-)}$-interfaces). Although $\delta$-containing TMD interfaces are present in the receptor complex, we focused solely on the $\alpha$-$\beta$ interfaces due to the confirmed existence of binding sites at these interfaces (Ernst *et al.*, 2005; Puthenkalam *et al.*, 2016; Laverty *et al.*, 2019), and our focus on benzodiazepine binding sites as potential binding sites for DS2 due to the structural resemblance between DS2 and zolpidem (Rostrup *et al.*, 2020). Key interacting residues were identified using homology models and pharmacologically characterized in well-established HEK cell-based assays using the $\alpha_4\beta_1\delta$ receptor as a model receptor, that has been carefully characterized in our hands (Falk-Petersen *et al.*, 2017; Dalby *et al.*, 2020; Falk-Petersen *et al.*, 2020).

**Investigation of the ECD $\alpha^{(+)}\delta^{(-)}$ Interface as the Site of Modulation by DS2**

First, using the homology model published in (Rostrup *et al.*, 2020), we studied the pocket located in the C-loop of the $\alpha^{(+)}\delta^{(-)}$ interface in the ECD of $\alpha_4\beta_1\delta$ receptors (Fig. 1A). From our previous docking into the model, we identified three potential key residues on the $\alpha_4^{(+)}$-side of the interface that could either interact directly with DS2 or were placed centrally
within the binding pocket: $\alpha_4$(F133), $\alpha_4$(R135) and $\alpha_4$(G191) (Fig. 1A). Additionally, five residues on the complementary $\delta^{\text{C}}$-interface were identified: $\delta$(E71), $\delta$(A73), $\delta$(F90), $\delta$(S155) and $\delta$(H204). The selected residues were mutated with the principle of removing potential interactions (mutation into alanine) and/or gradually decreasing the space in the binding pocket (mutation into various amino acid residues), thus expecting a reduced modulation by DS2 compared to WT. This resulted in seven different $\alpha_4$-subunit mutants and five different $\delta$-subunit mutants (Table S1+S2). Each of the mutated subunits were expressed in HEK cell lines to form $\alpha_4\beta_1\delta$ receptors and tested in the FMP assay, as single mutants. Whereas $\alpha_4$-mutants were simply co-transfected with WT $\beta_1$ into WT stable $\delta$-HEK293 Flp-In cells, each of the $\delta$-mutants were established as stable HEK293 Flp-In cell lines transfected with WT $\alpha_4\beta_1$ to transiently express $\alpha_4\beta_1\delta$. In general, extending the utility of this expression system from WT to mutated $\delta$-containing receptors is highly reliable and suitable for controlling expression and reliably studying these in some instances, cumbersome receptor subtypes (Karim et al., 2012).

All seven $\alpha_4$-mutant receptors were found to express functionally active receptors, and to respond to GABA with 2-3 times the potency observed for WT (Fig. 1B,C, Fig. S1, Table S1). The expression levels of $\alpha_4$(F133A/L)$\beta_1\delta$ and $\alpha_4$(G191E)$\beta_1\delta$ appeared lower as compared to WT, as the max $\Delta$RFU values were consistently reduced in all experiments (Fig. 1B). To characterize the sensitivity to DS2, it was applied together with a GABA EC$_{20}$ concentration, calculated for each mutant (Table 1, S2). Among the seven different $\alpha_4$-mutants, $\alpha_4$(F133A)$\beta_1\delta$ and $\alpha_4$(G191E)$\beta_1\delta$ showed no apparent or only small modulation by DS2, whereas the potency of DS2 at the other $\alpha_4$-subunit mutants was either unchanged ($\alpha_4$(F133L)$\beta_1\delta$, $\alpha_4$(R135A) and $\alpha_4$(G191A/L)$\beta_1\delta$) or slightly increased, ($\alpha_4$(R135H)$\beta_1\delta$), compared to WT (Fig. 1D-F, Table 1+ Fig. S1, Table S2). Interestingly,
only a single of the introduced mutations at α₁(F133) and α₄(G191) showed changed responses, which could not readily be explained.

As we and others have previously observed methodological limitations in the FMP assay (Wafford et al., 2009; Falk-Petersen et al., 2017), we suspected that the apparent lack of modulation could be due to sensitivity limitations. Thus, to follow up, the two mutants α₁(F133A) and α₄(G191E) were tested using whole-cell patch clamp electrophysiology. At both mutated receptors, DS2 modulated the GABA EC₂₀-induced currents in a concentration-dependent manner similar to WT or with even higher efficacy (Fig. 1G,H). Each of the five δ-subunit mutations were also tested in the FMP assay. These were all functional and displayed unchanged responsiveness to DS2 compared to WT (Fig. S2, Table S3,4).

Altogether, we conclude that the C-loop pocket in the ECD α₄(+δ(-)-interface is not the site responsible for the PAM effect of DS2.

Identification of the TMD α₄(+β₁(-) Interface as the Site of Modulation by DS2

Next, we looked into two pockets in the TMD αβ-interfaces (specifically involving TM2), as potential recognition sites for DS2, based on the hypothesis that diazepam and DS2 exhibit analogous binding sites in the TMD. Mutations in α₄β₁δ TMD pockets were suggested based on the cryo-EM structure of the human GABAₐR α₁β₃γ₂L (PDB-code: 6HUP) in combination with a sequence alignment due to the high (>90%) local sequence identity of the subunits within the TMD region of interest. The first pocket is located in the β₁(+α(-) interface in a site equivalent to the recently identified low-affinity binding site for diazepam (Laverty et al., 2019).

The mutations, β₁(S290F) on the β₁(+) side and α₄(L302Y) on the α₄(-) side were initially probed due to an apparent central positioning of the residues in the pocket and orientation towards diazepam in the cryo-EM structure (Fig. 2A). As a similar pocket is present at the
reverse subunit interface, we also included the corresponding mutations in the \( \alpha_4(+)\beta_1(-) \) interface, \( \alpha_4(S303L) \) on the \( \alpha_4(+) \) side and \( \beta_1(I289Q) \) on the \( \beta_1(-) \) side. However, as this pocket appears noticeably smaller than the \( \beta_1(+)\alpha_4(-) \) pocket, these were mutated into more flexible and less bulky residues. Additionally, to probe both proposed pockets simultaneously, we included the double mutant receptors \( \alpha_4(L302Y,S303L)\beta_1\delta \) and \( \alpha_4(L302Y,S290F)\beta_1\delta \). The introduced mutations were expected to revert hydrophilicity/hydrophobicity and introduce steric hindrance, and thus would be anticipated to decrease or altogether abolish the effect of DS2.

First, we show that all the single mutant receptors were GABA-responsive and thus functional in the FMP assay (Fig. 2B). The two \( \beta \)-mutants \( \beta_1(S290F) \) and \( \beta_1(I289Q) \), displayed 6.8 and 8.1 times increased GABA potencies, respectively, and the \( \alpha_4(S303L)\beta_1\delta \) mutant 2.9 times increased potency compared to WT (Fig. 2C, Table S5). Since the receptors were functional, we continued with the studies.

In the modulation experiments, we switched to DS2OMe, an analog of DS2 (L’Estrade et al., 2019) with the same pharmacological profile, because of both solubility issues with DS2 (described in the methods section) and the general sensitivity limitations observed in the FMP assay on the ECD mutants. First, we examined the modulation of GABA EC\(_{20}\) at mutations introduced in the \( \beta(+)\alpha(-) \) interface, equal to the low affinity diazepam binding site in the \( \gamma \)-containing receptor (Fig. 2D). These mutations did not affect the modulation by DS2OMe as both the \( \alpha_4\beta_1(S290F)\delta \) and \( \alpha_4(L302Y)\beta_1\delta \) mutant receptors had DS2OMe potencies similar to WT, although a small significant increase in efficacy for the \( \alpha_4(L302Y)\beta_1\delta \) mutant compared to WT was observed (**\( P=0.0063 \), two-tailed Welch’s \( t \)-test, response of 3 \( \mu \)M DS2OMe) (Fig. 2D,F, Table 2).

By contrast, when turning to the alternative \( \alpha_4(+)\beta_1(-) \) interface, we observed significant decreases in responsiveness to modulation by DS2OMe. The \( \alpha_4(S303L)\beta_1\delta \) receptor lacked
responsiveness to modulation by DS2OMe, and the β-mutant receptor, α4β1(I289Q)δ had a statistically significant 3.2 times reduction of the potency of DS2OMe compared to the WT receptor (Fig. 2E,F, Table 2). Additionally, as expected from the individual mutations, the double mutant receptor α4(L302Y,S303L)β1δ was not modulated by DS2OMe (Fig. S3, Table S6).

To verify the FMP results, we performed whole-cell patch-clamp electrophysiology recordings. Convincingly, we found no or very limited DS2 modulation of the GABA currents in the α4(S303L)β1δ and α4β1(I289Q)δ receptor (only 10 µM modulated the α4β1(I289Q)δ receptor by significantly increasing the GABA control current to 54.8% of the GABA I_max (**P=0.0063, two-tailed Welch’s t-test, adjusted, n=5-6) (Fig. 2G,H). Further, we included the double mutant receptor α4(S303L)β1(I289Q)δ, which was even less modulated by 10 µM DS2, amounting to 44% of the GABA I_max (*P=0.016, two-tailed Welch’s t-test, adjusted, n=6-9) (Fig. 2G,H, Tables S3 and S6). DS2OMe showed no modulation of the GABA response in either α4β1(I289Q)δ or α4(S303L)β1δ receptors, or the double mutant α4(S303L)β1(I289Q)δ receptor (Fig. S4).

Together, these results strongly advocate for the identified TMD α(+β(-) interface site as the site responsible for the modulatory action of DS2.

**Known GABA_A_R PAMs show Unchanged Modulation at DS2-insensitive Mutant Receptors**

To confirm that the mutant receptors with altered DS2 sensitivity were not overall compromised in their general PAM responsiveness, we tested etomidate (Hill-Venning et al., 1997) and AA29504 (Hoestgaard-Jensen et al., 2010; Olander et al., 2018) at both WT and the single mutants α4(S303L)β1δ and α4β1(I289Q)δ. In the FMP assay both compounds showed intact positive modulation of both mutants compared to WT. Potencies (pEC_{50}) of
etomidate were at WT αβδ determined to 5.11 (EC$_{50}$ 7.8 μM), and for the α$_4$(S303L)βδ and α$_4$β$_1$(I289Q)δ mutants to 5.21 and 5.03 (EC$_{50}$ 6.2 μM and 9.3 μM), respectively (Fig. 2I,J, Table 3) (NS, two-tailed Welch’s $t$-test, $n=3-4$). Further, AA29504 showed similar potentiation at the mutants and WT receptors (Fig. S5, Table S7), indicating that it does not mediate its effect through the same site as DS2, correlating with a proposed binding site for AA29504 in the TMD β$^{(+)}$α$^{(-)}$ interface (Olander et al., 2018).

**Induced-fit Docking of DS2 and DS2OMe Corroborates Mutational Results**

Guided by the mutational data confirming the molecular recognition site mediating the effect of DS2 and DS2OMe in the transmembrane part of the α$_4^{(+)}$β$_1^{(-)}$ subunit interface, we constructed a model of the modulator-receptor binding mode. Based on the structure of the desensitized α$_1$β$_3$γ$_2$L receptor bound to GABA and diazepam (Masiulis et al., 2019), we constructed a homology model of the α$_4$β$_1$ subunit interface into which DS2 and DS2OMe were fitted using induced-fit docking. Allowing residue side chains in the “empty” homology model to adapt to the modulators, we obtained very similar binding modes for the docked compounds (Fig. 3+ Fig. S5). The core scaffold binds with the amide carbonyl of DS2 showing a potential hydrogen bond to the hydroxy group of Ser303 in α$_4$. Using a backbone dependent rotamer library, we observe that the α$_4$(S303L) mutation removes the hydrogen bond and sterically blocks the binding site – providing an explanation for the observed lack of potentiation on this mutant (Fig. 3B). Ile289 in β$_1$ lines 4-fluorophenyl of DS2, contributing to the binding through substantial van der Waal contacts, and the I289Q mutation has a steric clash with DS2 (Fig. 3B). As for the α$_4$(S303L) mutant, these effects provide a possible explanation for the observed abolishment of potentiation at all but the highest concentration of DS2 and DS2OMe in our patch clamp experiments and the obtained binding mode thus concurs with the experimental results. Our previously published analogs
of DS2 show that there should be room for much larger substituents than the methoxy of DS2OMe as well as a bromo atom in the 5-position on the imidazol[1,2-a]pyridine scaffold (Rostrup et al., 2020). Thus, we provide further proof-of-concept for the predicted binding site by docking the recently published analog, Br-DS2OPh (Rostrup et al., 2020). This confirmed that the OPh substituent can fit the binding site in the homology model with only a minor shift in the binding mode (Fig. S6).

Discussion

From our experiments using systematic structural iterations and experimental validation of known and proposed binding sites, we here present the elusive DS2 interaction site as a distinct site, encompassing α4S303 and β1I289 residues in the TMD α4(+)/β1(−) interface of α4β1δ receptors. This novel site is similar in nature both to the low-affinity diazepam binding site identified in the cryo-EM structure of the α1β3γ2 receptor (Laverty et al., 2019) and the site for general anesthetics (e.g. etomidate) (Li et al., 2006) and the proposed binding site for AA29504 (Olander et al., 2018). Notably, the residues in the new DS2 site are located on the alternative inter-subunit interface α4(−)/β1(+), explaining why our mutations do not affect etomidate or AA29504 PAM activity. Indeed, it has been reported that pockets exist in all the TMD inter-subunit interfaces (Sieghart et al., 2012; Forman and Miller, 2016; Iorio et al., 2020), and that several known allosteric modulators can bind in these pockets (Olsen, 2018). This shows how different GABA_A receptors subtypes have evolved to include different functionally relevant allosteric sites.

The α4β1δ subtype was selected as model receptor in this study because of previous success with this for detailed and reliable molecular pharmacology examination (Falk-Petersen et al., 2017, 2020; Dalby et al., 2020). The expression of the δ-subunit relies on an in-house generated stable δ-HEK cell line and subsequent transfection with α and β subunits of choice,
including mutated subunits, to generate functional αβδ receptors that can be evaluated via measurements of membrane potential changes by fluorescence in the medium throughput FMP assay (Falk-Petersen et al., 2017). The combination of α4 and β1 subunits was selected, because α4 is often encountered together with δ in native receptors (Lee and Maguire, 2014), and because β1 conveniently does not lead to the formation of homomeric receptors in this system (Falk-Petersen et al., 2017). Indeed, using this setup we here demonstrate the measurement of both reliable GABA responses and various PAM effects at α4β1δ receptors. We also report the successful generation of several stable δ-mutant cell lines, thus underlining this expression system as a versatile methodological tool for studying δ-GABA_A molecular pharmacology in HEK cells with low variability. In cases of low expressing receptors as seen with some of the mutants examined here, we observed some discrepancies in the data obtained between FMP and patch-clamp electrophysiology. In this case the FMP assay, which measures overall changes in membrane potential, appears to have some limitations due to lower overall sensitivity especially in relation to efficacy of low-expressing mutants.

In our path to identifying the DS2 TMD interface binding site, we first examined one of the usual suspects; the ECD inter-subunit α4(+)-δ(-) interface, or the C-loop pocket (Jensen et al., 2013; Masiulis et al., 2019), as the site responsible for the PAM effect of DS2. Although the existence of this pocket has previously been debated (Wafford et al., 2009; Jensen et al., 2013; Ahring et al., 2016), we included it due to an observed structural resemblance between DS2 and the benzodiazepine binding site ligand, zolpidem, which could suggest a potentially shared benzodiazepine-like binding site. We can now refute this hypothesis, also corroborated by our recent structure-activity-relationship study of DS2 analogs targeting this site (Rostrup et al., 2020). Incidentally, one of these analogs (Br-DS2OPh), designed to bind in the ECD α(+)-δ(-) interface, fits well into the identified DS2 TMD pocket in the α4(+)-β1(-)
interface, showing the importance of experimental validation of binding site hypotheses based on molecular modeling. This further identifies Br-DS2OPh as a useful DS2 analog for future studies.

From our data it is evident that the δ-subunit is not directly involved in the modulation by DS2, questioning what determines the δ-selective profile of the compounds. This is in accordance with previous data by Yakoub et al. who found that DS2 is capable of modulating receptors (in particular binary α6β3 receptors) that do not contain a δ-subunit (Yakoub et al., 2018). It is plausible that this is a matter of functional selectivity, similar to that observed for the super agonist THIP (gaboxadol), in which case binding in a highly conserved α-β interface gives rise to 10 times higher potency at the δ-containing receptors compared to both γ-containing and binary αβ receptors (Stórustovu and Ebert, 2006; Mortensen et al., 2010; Falk-Petersen et al., 2017). Also PAMs, such as neurosteroids, have been found to display functional selectively at δ-containing receptors (Stell et al., 2003; Ahring et al., 2016), supposedly because GABA itself is only a partial agonist leaving room for further activation (Dalby et al., 2020). Now, having a homology model of the confirmed DS2 binding site, the next step is to use this for structure-based drug design of DS2-related analogs or a radiolabeled analog for further validation of the binding site. Already, we have shown that classical medicinal chemistry approaches can improve both potency and selectivity of DS2 (Rostrup et al., 2020), and potentially brain permeability (L’Estrade et al., 2019). However, in line with already reported PAM effects, DS2’s functional activity is most effective at δ-containing subtypes (Jensen et al., 2013; Ahring et al., 2016; Yakoub et al., 2018), due to yet, unknown factors. Ultimately, a cryo-EM structure in complex with one of the DS2 analogs would map the binding pocket including additional molecular interaction points and discern potential differences among subtypes.
In conclusion, our identification of the long-sought-after DS2 interaction site in the $\alpha_4\beta_1\delta$ receptor may promote new insights into this highly important drug target class of $\delta$-containing receptors suffering a general lack of selective tool compounds. Novel $\delta$-selective analogs will aid to improve our understanding of the physiological and pathophysiological role of $\delta$-containing receptors. Such compounds may therefore potentially serve as leads for future rational drug development to treat the vast majority of neurological disorders with dysregulated tonic inhibition as well as targeting conditions involving $\delta$-containing GABA$\text{A}$ receptors in the periphery such as inflammation and immune disorders.

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Authorship Contributions

Participated in research design: Falk-Petersen, Rostrup, Harpsøe, Gloriam, Frølund, Wellendorph

Conducted experiments: Falk-Petersen, Rostrup, Löffler, Buchleithner, Harpsøe.

Contributed new reagents: Rostrup, Frølund

Performed data analysis: Falk-Petersen, Rostrup, Löffler, Buchleithner, Harpsøe, Wellendorph

Wrote or contributed to the writing of the manuscript: Falk-Petersen, Harpsøe, Wellendorph

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Ahring PK, Bang LH, Jensen ML, Strobaek D, Hartiadi LY, Chebib M, and Absalom N (2016) A pharmacological assessment of agonists and modulators at $\alpha_4\beta_2\gamma_2$ and $\alpha_4\beta_2\delta$


(DS2) at human recombinant and rodent native GABA<sub>A</sub> receptors. *Br J Pharmacol* **168**:1118–1132.


**Footnotes**

a) Financial support:

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b) Thesis reference:

Part of this work was described in the PhD thesis by C.B.F.-P. (2020): Pharmacological insight into GABA<sub>A</sub> receptors with focus on β<sub>1</sub>-containing extrasynaptic subtypes, the Faculty of Health and Medical Sciences, University of Copenhagen, Denmark.

**Legends for figures**

**Figure 1.** Investigation of the potential ECD α<sub>4</sub>(+)-δ(-)-interface binding site. **A** Model of the α<sub>4</sub>β<sub>1</sub>δ receptor with zoom-in on mutated residues in the α<sub>4</sub>(+)-δ(-)-interface (built on the cryo-EM structure of α<sub>1</sub>β<sub>2γ2</sub> (PBD code: 6D6T)). **B** Single representative GABA concentration-
response curves for \( \alpha_4 \)-mutant receptors (means ± S.D., technical triplicates), and C bar diagram of pooled pEC\(_{50} \) values (means with 95% CI, each point representing an independent replicate \((n=4-8)\). D-E Concentration-response curves of the modulation of GABA EC\(_{20} \) by DS2 at \( \alpha_4 \)-mutant receptors (normalized means±SD, technical triplicates), and F bar diagram of pooled pEC\(_{50} \) values (means with 95% CI, each point representing an independent replicate \((n=3-5)\). G Single cell representative current traces from whole-cell patch-clamp electrophysiology recordings of the modulation of GABA EC\(_{20} \) induced currents by DS2 at WT \( \alpha_4\beta_1\delta \) and mutants \( \alpha_4(F133A)\beta_1\delta \) and \( \alpha_4(G191E)\beta_1\delta \) receptors. H Bar diagram summarizing the modulation by DS2 of \( \alpha_4 \) mutants \( \alpha_4(F133A), \alpha_4(G191E) \) cf. WT (note the broken y-axis). Currents were normalized to the maximum GABA current and presented as mean % \( \text{I/I}_{\text{max}} \) with 95% CI from minimum two independent transfections \((n=5-7)\). For C,F,H: Statistical analysis was performed using two-sided Welch’s t-test compared to WT (C,F) or control current (H) and adjusted for multiple testing using the Original FDR method of Benjamini and Hochberg with a discovery rate of 0.05. Statistical significance *P<0.05, **P<0.01, ***P<0.001.

**Figure 2.** Identification of the TMD \( \alpha_4^{(+)}\beta_1^{(-)} \) site mediating modulation by DS2. A Model of the \( \alpha_4\beta_1\delta \) receptor with the TMD \( \beta_1^{(+)}\alpha_4^{(-)} \) and \( \alpha_4^{(+)}\beta_1^{(-)} \) interface mutants highlighted in the zoom-in (build on the cryo-EM structure of the \( \alpha_1\beta_3\gamma_2 \) receptor (PBD code: 6HUP). B Single representative GABA concentration-response curves from WT \( \alpha_4\beta_1\delta \) and TMD \( \alpha\beta \)-interface mutants (means ± S.D., technical triplicates), and C bar diagram showing pooled pEC\(_{50} \) values (means with 95% CI \((n=4-11)\). Modulation of GABA EC\(_{20} \) by DS2OMe at D the \( \beta_1^{(+)}\alpha_4^{(-)} \) and E the \( \alpha_4^{(+)}\beta_1^{(-)} \) interface mutants. Data are representative curves from a single experiment with means ± S.D. of data normalized to GABA\(_{\text{max}} \). F Bar diagram showing pooled pEC\(_{50} \) values (means with 95% CI \((n=4-7)\). G Single cell current traces from DS2
modulation of GABA EC_{20} at WT and \( \alpha_{4}^{(+)}\beta_{1}^{(-)} \) interface mutants. **H** Bar diagram summarizing the DS2 modulation of \( \alpha_{4}^{(+)}\beta_{1}^{(-)} \) interface single and double mutants in whole-cell patch-clamp electrophysiology. Currents are normalized to the GABA_{max} current and are given as mean \% I/I_{max} with 95% CI (n=5-16). **I** Single representative concentration-response curves of the modulation of GABA EC_{20} by etomidate at the \( \alpha_{4}^{(+)}\beta_{1}^{(-)} \) interface mutants, and **J** pooled pEC_{50} values (means with 95% CI with symbols representing values from independent experiments (n=4-5)). For **C,F,H,J**: Statistical analysis was performed using two-sided Welch’s \( t \)-test compared to WT (C,F,J) or control current (H) and adjusted for multiple testing using the Original FDR method of Benjamini and Hochberg with a discovery rate of 0.05. Statistical significance *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

**Figure 3.** Binding model of DS2 in the TMD \( \alpha_{4}^{(+)}\beta_{1}^{(-)} \) subunit interface. Visualization of DS2 (sticks and grey carbon atoms) in the TMD interface between the \( \alpha_{4} \) (blue cartoon and carbon atoms) and \( \beta_{1} \) (magenta cartoon and carbon atoms) GABA_{A} subunits, showing: **A** Residues with side chain atoms within 5 Å of DS2 are shown as lines highlighting the two important residues, \( \alpha_{4} \)S303 and \( \beta_{1} \)I289, as sticks. The binding cavity is depicted as the vdW surface (grey and transparent) of the same residues and hydrogen bonds between DS2 and the receptor represented as yellow dotted lines. **B** *In silico* representation of the \( \alpha_{4} \)S303L and \( \beta_{1} \)I289Q mutations (inserted residues as sticks with yellow carbon atoms) showing predicted steric clashes with DS2 as red disks explaining the hampered/abolished positive modulation of DS with these mutations. Figure prepared with the PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.
### Tables

**Table 1.** Potencies of DS2 at WT and ECD α₄-mutant receptors determined in the FMP assay.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>DS2 (PAM) EC₅₀ (µM)</th>
<th>pEC₅₀ (95% CI), n</th>
<th>difference pEC₅₀ [95% CI]</th>
<th>P-value</th>
<th>Adjusted P-value</th>
<th>GABA EC₂₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.97</td>
<td>6.01 (5.77;6.25), 4</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>α₄(F133A)β₁δ</td>
<td>-</td>
<td>n=5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.12</td>
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<tr>
<td>α₄(F133L)β₁δ</td>
<td>1.29</td>
<td>5.89 (5.24;6.54), 3</td>
<td>-0.12 [-0.65;0.42]</td>
<td>0.53</td>
<td>0.7857</td>
<td>0.12</td>
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<tr>
<td>α₄(R135A)β₁δ</td>
<td>1.41</td>
<td>5.85 (5.76;5.95), 4</td>
<td>-0.15 [-0.38;0.075]</td>
<td>0.14</td>
<td>0.4500</td>
<td>0.06</td>
</tr>
<tr>
<td>α₄(G191A)β₁δ</td>
<td>2.04</td>
<td>5.69 (5.77;6.25), 4</td>
<td>-0.32 [-0.66;0.02]</td>
<td>0.061</td>
<td>0.3050</td>
<td>0.12</td>
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<tr>
<td>α₄(G191E)β₁δ</td>
<td>-</td>
<td>n=5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.06</td>
</tr>
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*α* not able to fit, but a small potentiation seen for concentrations higher than 1 µM. EC₂₀; calculated GABA concentration co-applied with the PAM. Statistical analysis: two-tailed Welch’s *t*-test compared to WT, adjusted for multiple comparison using the original FDR (Benjamini and Hochberg) method with discovery rate of 0.05.
<table>
<thead>
<tr>
<th>Receptor</th>
<th>DS2OMe (PAM)</th>
<th>Difference pEC(_{50})</th>
<th>P-value</th>
<th>Adjusted P-value</th>
<th>GABA</th>
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<tbody>
<tr>
<td></td>
<td>EC(_{50}) (µM)</td>
<td>pEC(_{50}) (95% CI), n</td>
<td>[95% CI]</td>
<td>P-value</td>
<td>EC(_{20}) (µM)</td>
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<tr>
<td>WT</td>
<td>0.50</td>
<td>6.30 (5.9;6.7), 5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α(_4L302Y)β(_1 δ)</td>
<td>0.35</td>
<td>6.46 (6.3;6.6), 5</td>
<td>0.17 [-0.19;0.52]</td>
<td>0.29</td>
<td>0.310</td>
</tr>
<tr>
<td>α(_4S303L)β(_1 δ)</td>
<td>-</td>
<td>n=3*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α(_4β1I289Q δ)</td>
<td>1.58</td>
<td>5.80 (5.6;6.0), 7</td>
<td>-0.49 [-0.85;-0.12]</td>
<td>0.015</td>
<td>0.045*</td>
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<td>α(_4β1S290F δ)</td>
<td>0.35</td>
<td>6.46 (6.3;6.7), 4</td>
<td>0.16 [-0.20;0.52]</td>
<td>0.31</td>
<td>0.310</td>
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* no apparent potentiation (concentration range from 0.01 µM to 10 µM DS2OMe). EC\(_{20}\); calculated GABA concentration co-applied with the PAM. Statistical analysis; Two-tailed Welch’s t-test compared to WT adjusted for multiple comparison using the original FDR method of Benjamini and Hochberg with a discovery rate of 0.05. Significance level; *P<0.05.
Table 3. Potency of etomidate at $\alpha_4^{(+)}\beta_1^{(-)}$ TMD mutants determined in the FMP assay

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Etomidate (PAM) EC$_{50}$ (µM)</th>
<th>pEC$_{50}$ (95% CI), n</th>
<th>Difference pEC$_{50}$</th>
<th>P-value</th>
<th>Adjusted P-value</th>
<th>GABA EC$_{20}$</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>7.8</td>
<td>5.11 (5.1;7.4), 5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.06</td>
</tr>
<tr>
<td>$\alpha_4$S303L$\beta_1$$\delta$</td>
<td>6.2</td>
<td>5.21 (6.0;7.2), 4</td>
<td>-0.10 [-1.3;0.60]</td>
<td>0.36</td>
<td>0.66</td>
<td>0.02</td>
</tr>
<tr>
<td>$\alpha_4$$\beta_1$I289Q$\delta$</td>
<td>9.3</td>
<td>5.03 (6.1;6.2), 5</td>
<td>-0.07 [-1.4;1.31]</td>
<td>0.66</td>
<td>0.66</td>
<td>0.007</td>
</tr>
</tbody>
</table>

EC$_{20}$: calculated GABA concentration co-applied with the PAM. Statistical analysis; two-tailed Welch’s $t$-test compared to WT and adjusted for multiple testing using the original FDR method of Benjamini and Hochberg with a discovery rate of 0.05.
Molecular Determinants underlying Delta Selective Compound 2 (DS2) Activity at δ-Containing GABA_{A} Receptors


Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark.

This PDF file includes:

- Figures S1 to S5
- Tables S1 to S7
**Fig. S1.** Characterization of selected α4 ECD mutants in the FMP assay. 

**A** Representative GABA concentration-response curves shown as means ± S.D., and **B** bar diagram showing pooled GABA pEC₅₀ values (means with 95% CI, n=3-7). **C** Representative curves of the modulation of GABA EC₂⁰ by DS2 shown as normalized means ± S.D. and **D** bar diagram showing pooled pEC₅₀ values (means with 95% CI, n=3-4). Statistical analysis of pEC₅₀ values was performed using two-tailed Welch’s t-test compared to WT and adjusted for multiple testing using the original FDR method of Benjamini and Hochberg with a discovery rate of 0.05. Statistical significance *P<0.05.
Fig. S2. Characterization of ECD δ-subunit mutant receptors in the FMP assay. A Representative GABA concentration-response curves shown as means ± S.D. and B bar diagram showing pooled GABA pEC\textsubscript{50} values (means with 95% CI, n=3-8). C Representative curves of the modulation of GABA EC\textsubscript{20} by DS2 shown as normalized means ± S.D. and D bar diagram showing pooled pEC\textsubscript{50} values (means with 95% CI, n=4-5). Statistical analysis of pEC\textsubscript{50} values was performed using two-tailed Welch's t-test compared to WT and adjusted for multiple testing using the original FDR method of Benjamini and Hochberg with a discovery rate of 0.05. Statistical significance *P<0.05 and ***P<0.001.
Fig. S3. Characterization of TMD double mutants in the FMP assay. A Representative GABA concentration-response curves (means ± S.D.) at selected TMD double mutants and B bar diagram summarizing the pooled GABA pEC\textsubscript{50} values (means with 95% CI, n=5-11). The mutant α4β1(1289Q,S290F)δ could not be fitted to a sigmoidal function due to low responses to GABA (n=3). C Decrease of the baseline signal by Picrotoxin at the α4β1(1289Q,F290F)δ mutant, indicating constitutive activity (representative curve shown as normalized means ± S.D., (n=2)). D Representative curve of DS2OMe modulation at α4(L302Y,S303L)β1δ, means ± S.D. (n=3). Statistical analysis of pEC\textsubscript{50} values was performed using two-tailed Welch’s t-test compared to WT and adjusted for multiple testing using the original FDR method of Benjamini and Hochberg with a discovery rate of 0.05. Significance levels **P<0.01 and ***P<0.001.
Fig. S4. Characterization of DS2OMe modulation at WT $\alpha_4\beta_1\delta$, the TMD $\alpha_4^{(*)}\beta_1^{(*)}$ double mutant $\alpha_4$(S303L)$\beta_1$(I289Q)$\delta$ and single mutants in whole cell patch clamp electrophysiology. A and C show representative cell current traces for the modulation. In B and D are shown bar diagrams of the pooled normalized currents given as means with 95% CI from 5-16 cells. Statistical analysis was performed using two-tailed Welch’s $t$-test compared to control current and adjusted for multiple testing using the original FDR method of Benjamini and Hochberg with a discovery rate of 0.05. Significance levels **P<0.01, ***P<0.001 and ****P<0.0001.
Fig. S5. Binding model of DS2OMe and Br-DS2OPh in the TMD $\alpha_4\beta_1$ subunit interface. Potential binding mode of, A, DS2OMe and, B, Br-DS2OPh as sticks and grey carbon atoms in the TMD interface between the $\alpha_4$ (blue cartoon and carbon atoms) and $\beta_1$ (magenta cartoon and carbon atoms) GABA$_A$ subunits. Residues with side chain atoms within 5 Å of the compounds are shown as lines highlighting the two important residues, $\alpha_4$S303 and $\beta_1$I289, as sticks. Hydrogen bonds to receptor are represented as yellow dotted lines. Figure prepared with the PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.
Table S1. GABA potencies at WT and ECD α4-mutant receptors.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>GABA (agonist) EC₅₀ (µM), pEC₅₀±S.E.M., n</th>
<th>95% CI pEC₅₀</th>
<th>Difference pEC₅₀ mean [95% CI]</th>
<th>P-value</th>
<th>Adjusted P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.17 (6.77± 0.038, 8)</td>
<td>[6.68;6.86]</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α₄F133Aβ₁δ</td>
<td>0.42 (6.38 ± 0.097, 4)</td>
<td>[6.07;6.69]</td>
<td>-0.39 [-0.69;-0.10]</td>
<td>0.020</td>
<td>0.071</td>
</tr>
<tr>
<td>α₄F133Lβ₁δ</td>
<td>0.33 (6.48 ± 0.091, 5)</td>
<td>[6.32;6.65]</td>
<td>-0.29 [-0.44;-0.14]</td>
<td>0.0034</td>
<td>0.024*</td>
</tr>
<tr>
<td>α₄R135Aβ₁δ</td>
<td>0.18 (6.74 ± 0.087, 5)</td>
<td>[6.50;7.98]</td>
<td>-0.03 [-0.27;0.20]</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>α₄R135Hβ₁δ</td>
<td>0.11 (6.97 ± 0.066, 3)</td>
<td>[6.68;7.26]</td>
<td>-0.20 [-0.026;0.43]</td>
<td>0.069</td>
<td>0.11</td>
</tr>
<tr>
<td>α₄G191Aβ₁δ</td>
<td>0.18 (6.74 ± 0.090, 5)</td>
<td>[6.48;6.98]</td>
<td>-0.04 [-0.28;0.21]</td>
<td>0.71</td>
<td>0.75</td>
</tr>
<tr>
<td>α₄G191Eβ₁δ</td>
<td>0.27 (6.57 ± 0.082, 4)</td>
<td>[6.31;6.83]</td>
<td>-0.21 [-0.45;0.038]</td>
<td>0.080</td>
<td>0.11</td>
</tr>
<tr>
<td>α₄G191Lβ₁δ</td>
<td>0.18 (6.97 ± 0.066, 4)</td>
<td>[6.69;7.25]</td>
<td>0.20 [-0.026;0.42]</td>
<td>0.069</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Statistical analysis was performed using two-tailed Welch’s t-test compared to WT and adjusted for multiple testing using the original FDR method of Benjamini and Hochberg with a discovery rate of 0.05. Significance level *P<0.05. Data was obtained in the FMP assay.
Table S2. Potencies of the PAM DS2 at WT and selected ECD α4-mutant receptors.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>DS2 (PAM) EC_{50} (µM)</th>
<th>pEC_{50} ±S.E.M., n</th>
<th>95% CI pEC_{50} mean [95% CI]</th>
<th>Difference pEC_{50} mean [95% CI]</th>
<th>P-value</th>
<th>Adjusted P-value</th>
<th>EC_{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.97</td>
<td>(6.01 ± 0.076, 4)</td>
<td>[5.8;6.3]</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.06</td>
</tr>
<tr>
<td>α_4(R135H)β_3δ</td>
<td>0.31</td>
<td>(6.51 ± 0.050, 3)</td>
<td>[6.3;6.7]</td>
<td>-0.50 [0.27;0.73]</td>
<td>0.003</td>
<td>0.030*</td>
<td>0.06</td>
</tr>
<tr>
<td>α_4(G191L)β_3δ</td>
<td>1.15</td>
<td>(5.94 ± 0.10, 3)</td>
<td>[5.5;6.4]</td>
<td>-0.07 [-0.41;0.28]</td>
<td>0.63</td>
<td>0.79</td>
<td>0.06</td>
</tr>
</tbody>
</table>

EC_{20}; GABA concentration coapplied with the PAM. Statistical analysis; two-tailed Welch’s t-test compared to WT, adjusted for multiple comparison using the original FDR (Benjamini and Hochberg) method with discovery rate of 0.05. EC_{20}; modulated GABA concentration. Data was obtained in the FMP assay.
**Table S3.** GABA potencies at WT and ECD δ-mutant receptors.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>GABA (Agonist) EC₅₀ (µM), pEC₅₀±S.E.M., n</th>
<th>95% CI pEC₅₀</th>
<th>Difference pEC₅₀ mean [95% CI]</th>
<th>P-value</th>
<th>Adjusted P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.17 (6.77± 0.038, 8) [6.68;6.86]</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α₄δ₁(δ(E71L))</td>
<td>0.26 (6.59 ± 0.034, 3) [6.44;6.74]</td>
<td>-0.18 [-0.31;-0.06]</td>
<td>0.009</td>
<td>0.023*</td>
<td></td>
</tr>
<tr>
<td>α₄δ₁(δ(A73N))</td>
<td>0.31 (6.51 ± 0.011, 3) [6.46;6.56]</td>
<td>-0.26 [-0.35;-0.17]</td>
<td>0.0002</td>
<td>0.0010***</td>
<td></td>
</tr>
<tr>
<td>α₄δ₁(δ(F90A))</td>
<td>0.19 (6.71 ± 0.018, 3) [6.63;6.79]</td>
<td>-0.07 [-0.16;0.03]</td>
<td>0.16</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>α₄δ₁(δ(S155Q))</td>
<td>0.23 (6.64 ± 0.079, 3) [6.29;6.99]</td>
<td>-0.14 [-0.42;0.15]</td>
<td>0.23</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>α₄δ₁(δ(H204A))</td>
<td>0.19 (6.71 ± 0.097, 3) [6.29;7.14]</td>
<td>-0.06 [-0.42;0.30]</td>
<td>0.62</td>
<td>0.62</td>
<td></td>
</tr>
</tbody>
</table>

Statistical analysis; two-tailed Welch’s t-test compared to WT and adjusted for multiple testing using the original FDR method of Benjamini and Hochberg with a discovery rate of 0.05. Data was obtained in the FMP assay.
### Table S4. DS2 potencies at WT and ECD δ-mutant receptors.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>DS2 (PAM)</th>
<th>95% CI</th>
<th>Difference pEC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>P-value</th>
<th>Adjusted P-value</th>
<th>EC&lt;sub&gt;20&lt;/sub&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.97</td>
<td>(6.01 ± 0.076, 4)</td>
<td>[5.8;6.3]</td>
<td>-</td>
<td>-</td>
<td>0.06</td>
</tr>
<tr>
<td>α&lt;sub&gt;4&lt;/sub&gt;β&lt;sub&gt;1&lt;/sub&gt;δ(E71L)</td>
<td>1.20</td>
<td>(5.92 ± 0.13, 5)</td>
<td>[5.6;6.3]</td>
<td>-0.09 [-0.44;0.26]</td>
<td>0.55</td>
<td>0.79</td>
</tr>
<tr>
<td>α&lt;sub&gt;4&lt;/sub&gt;β&lt;sub&gt;1&lt;/sub&gt;δ(A73N)</td>
<td>1.10</td>
<td>(5.96 ± 0.14, 4)</td>
<td>[5.7;6.4]</td>
<td>0.04 [-0.34;0.43]</td>
<td>0.79</td>
<td>0.87</td>
</tr>
<tr>
<td>α&lt;sub&gt;4&lt;/sub&gt;β&lt;sub&gt;1&lt;/sub&gt;δ(F90A)</td>
<td>1.35</td>
<td>(5.87 ± 0.15, 4)</td>
<td>[5.4;6.4]</td>
<td>-0.13 [-0.60;0.32]</td>
<td>0.46</td>
<td>0.79</td>
</tr>
<tr>
<td>α&lt;sub&gt;4&lt;/sub&gt;β&lt;sub&gt;1&lt;/sub&gt;δ(S155Q)</td>
<td>0.89</td>
<td>(6.05 ± 0.26, 4)</td>
<td>[5.2;6.9]</td>
<td>-0.05 [-0.76;0.85]</td>
<td>0.87</td>
<td>0.87</td>
</tr>
<tr>
<td>α&lt;sub&gt;4&lt;/sub&gt;β&lt;sub&gt;1&lt;/sub&gt;δ(H204A)</td>
<td>1.51</td>
<td>(5.82 ± 0.096, 4)</td>
<td>[5.5;6.1]</td>
<td>-0.19 [-0.48;0.11]</td>
<td>0.18</td>
<td>0.45</td>
</tr>
</tbody>
</table>

**EC<sub>20</sub>**, GABA concentration coapplied with the PAM. Statistical analysis; two-tailed Welch’s t-test, adjusted for multiple comparison using the original FDR method of Benjamini and Hochberg with a discovery rate of 0.05. Data is from the FMP assay.
Table S5. GABA potencies at WT and TMD mutant receptors.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>GABA (Agonist)</th>
<th>95% CI pEC50</th>
<th>Difference pEC50 mean [95% CI]</th>
<th>P-value</th>
<th>Adjusted P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50 (µM), pEC50 ± S.E.M., n</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>0.081</td>
<td>[7.09 ± 0.056, 11]</td>
<td>[7.0;7.2]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α4(L302Y)β1δ</td>
<td>0.058</td>
<td>[7.24 ± 0.12, 4]</td>
<td>[6.9; 7.6]</td>
<td>0.14 [-0.20;0.49]</td>
<td>0.31</td>
</tr>
<tr>
<td>α4(S303L)β1δ</td>
<td>0.029</td>
<td>[7.54 ± 0.091, 6]</td>
<td>[7.3; 7.8]</td>
<td>0.44 [0.20;0.69]</td>
<td>0.0024</td>
</tr>
<tr>
<td>α4β1(I289Q)δ</td>
<td>0.010</td>
<td>[7.84 ± 0.037, 7]</td>
<td>[7.8; 7.9]</td>
<td>0.74 [0.60;0.89]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>α4β1(S290F)δ</td>
<td>0.012</td>
<td>[7.98 ± 0.11, 6]</td>
<td>[7.7; 8.3]</td>
<td>0.87 [0.60;1.17]</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Statistical analysis; two-tailed Welch’s t-test, adjusted for multiple comparison using the original FDR method of Benjamini and Hochberg with a discovery rate of 0.05. EC20 is the used GABA concentration. Data is from the FMP assay.
<table>
<thead>
<tr>
<th>Receptor</th>
<th>GABA (agonist)</th>
<th>95% CI</th>
<th>Difference pEC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (µM), pEC&lt;sub&gt;50&lt;/sub&gt; ± S.E.M., n</td>
<td>pEC&lt;sub&gt;50&lt;/sub&gt; mean [95% CI]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>0.081 (7.09±0.056, 11)</td>
<td>[7.0;7.2]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α&lt;sub&gt;4&lt;/sub&gt;δ&lt;sub&gt;4(L302Y,S303L)β&lt;sub&gt;1&lt;/sub&gt;δ</td>
<td>0.027 (7.57 ± 0.094, 5)</td>
<td>[7.3;7.8]</td>
<td>0.48 [0.22;0.74]</td>
<td>0.003**</td>
</tr>
<tr>
<td>α&lt;sub&gt;4β&lt;sub&gt;1(289Q,S290F)δ</td>
<td>-</td>
<td>n = 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α&lt;sub&gt;4δ&lt;sub&gt;4(S303L)β&lt;sub&gt;1(290Q)δ</td>
<td>0.016 (7.80 ± 0.067, 5)</td>
<td>[7.6;8.0]</td>
<td>0.70 [0.50;0.90]</td>
<td>&gt;0.0001***</td>
</tr>
</tbody>
</table>

<sup>a</sup>No response to GABA. Statistical analysis was performed using two-tailed Welch’s t-test. Data was obtained in the FMP assay.
Table S7. AA29504 potencies at TMD $\alpha_4^{(*)}\beta_1^{(*)}$ interface mutants.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>AA29504 (PAM) EC$<em>{50}$(µM), pEC$</em>{50}$ ± S.E.M., n</th>
<th>95% CI pEC$_{50}$</th>
<th>Difference pEC$_{50}$ mean [95% CI]</th>
<th>P-value</th>
<th>EC$_{20}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.55 (6.26 ± 0.27, 3) [5.0;5.3]</td>
<td>-</td>
<td>-</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>$\alpha_4$(S303L)$\beta_1$$\delta$</td>
<td>0.25 (6.61 ± 0.20, 4) [4.8;5.6]</td>
<td>-0.35 [-0.31;0.51]</td>
<td>0.53 [0.02]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha_4$β1(289Q)$\delta$</td>
<td>0.76 (6.12 ± 0.015, 3) [4.7;5.3]</td>
<td>0.14 [-0.38;0.23]</td>
<td>0.58 [0.007]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

EC$_{20}$: GABA concentration coapplied with the PAM. Statistical analysis was performed using two-tailed Welch’s t-test. Data was obtained in the FMP assay.