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Multiple structural features of steroids mediate subtype-selective effects on human α4β3δ $GABA_A$ receptors

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Abstract

Neurosteroids have been shown to mediate some of their physiological effects via a modulatory site on type A inhibitory γ-aminobutyric acid ($GABA_A$) receptors. In particular, recent evidence has implicated selective potentiation of the δ subunit of $GABA_A$ receptors as an important mediator of in vitro and in vivo neurosteroid activity. However, this has been demonstrated for only a very small number of steroids, so both the generality of this finding, and the structural features of steroids which mediate functional δ-selectivity, are unclear. We have used a potentiometric assay based on fluorescence resonance energy transfer to measure GABA-activated responses in L(tk−) cells stably transfected with human $GABA_A$ receptor α4β3δ and α4β3γ2 receptor subtypes. A set of 28 steroids were evaluated on these subtypes to characterise their functional potency and efficacy in modulating GABA responses. For most compounds there was a clear separation of their efficacy profiles between the receptor subtypes, with a substantially larger maximal response at the α4β3δ receptor. 5β-Pregnan-3β-ol-20-one, 5β-pregnane-3α,20β-diol and 5β-pregnane-3α,17α-diol-11,20-dione showed particularly high efficacy for α4β3δ. No compounds were identified that simply inhibited responses at δ-containing receptors. However, 5β-pregnane-3α,17α,20β-triol, prednisolone 21-acetate, 4-pregnene-17α,20α-diol-3-one-20-acetate, 4-pregnen-20α-ol-3-one, and 5β-pregnane-3α,17α,21-triol-20-one inhibited, though did not abolish, GABA responses at the α4β3γ2 subtype, while evoking modest-amplitude potentiation of α4β3δ responses. Molecular modelling on this compound series using principal components analysis indicates that several structural features of steroids underlie their relative functional selectivity for potentiation of δ-containing $GABA_A$ receptors.

Keywords: Neurosteriods; Potentiometric assay; Neurotransmitter; GABA; Delta subunit

1. Introduction

The principal inhibitory neurotransmitter in the central nervous system is γ-aminobutyric acid (GABA). Type A GABA ($GABA_A$) receptors are rapid acting ligand-gated Cl^- channels which consist of different combinations of subunits assembled from a group including α_{1-6}, β_{1-4},γ_{1-3}, δ, ε, π, $\rho_{1-3}$and θ [1–4]. The specific subunit combination determines the biophysical and pharmacological properties of the receptors. For the most part, $GABA_A$receptors consist of a pentameric arrangement of two α subunits, two β subunits and either a γ or δ subunit. Several drugs exert their effects through these receptors including clinically important benzodiazepines, sedative and anaesthetic barbiturates, and steroids [4]. The density and distribution within the central nervous system of the subunit combinations is variable. The α1 subunit in combination with the β2 and γ2 subunits is the most abundant and is located in almost every region, as demonstrated by in situ and immunocytochemical techniques [5,6]. In addition, the γ2 subunit is the most abundant of the γ subunits in the brain, and it can be further differentiated into short (γ2S) and long (γ2L) subunits.

α4 and δ subunit distributions are relatively restricted, forming for example a significant proportion of $GABA_A$ receptors in the hippocampus and thalamus [7,8]. The $GABA_A$ receptors containing α4 subunits appear to have differing properties to other receptor subtypes, e.g., those containing α4, β and γ2 subunits have a distinct atypical

Abbreviations: CC2-DMPE, CC2-dimyristoyl phosphatidylethanolamine; $DiSBAC_2$ (3), bis-(1,3-diethylthiobarbituric acid) trimethine oxonol; VIPR, voltage/ion probe reader; PCA, principal components analysis; PC, principal component

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benzodiazepine binding site, and those containing α4, β and δ subunits do not have a benzodiazepine site at all [7,9,10]. The δ subunits convey a unique pharmacology to $GABA_A$ receptors, by increasing GABA affinity and reducing receptor desensitisation, which is consistent with an apparent extrasynaptic role in tonic inhibition [11–14]. $GABA_A$ receptors containing a δ subunit also exhibit a unique response to the GABA site agonist THIP, which at these receptors elicits a maximum current greater than GABA itself [15].

Neurosteroids act as allosteric modulators of the $GABA_A$ receptor, possibly at a binding site on the β subunit [16,17]. Steroid modulation of the GABA response requires a β subunit, however, the presence of a γ subunit is not required, unlike the benzodiazepine site where this is a prerequisite [18]. Neurosteroids are synthesised from cholesterol in the central nervous system, occurring in both glial and neuronal cells, by a series of enzymatic steps [19]. They have been implicated in premenstrual syndrome [20], cognitive and psychiatric dysfunctions, neuroprotection [21], and $GABA_A$ receptor plasticity [22,23]. Withdrawal from chronic exogenous neurosteroids may be associated with increased seizure susceptibility due to alterations in expression of $GABA_A$ receptor subunits [22]. Neurosteroid sensitivity has also been observed to be attenuated in δ knockout mice [24].

$GABA_A$ receptors containing α4 and δ, on account of their restricted location within the central nervous system [7,8], potential role in disease states [22,25,26], and unique electrophysiological properties are therefore an interesting target to further evaluate. Recent studies from this and other labs have indicated that, in vitro, δ-containing $GABA_A$ receptors have higher sensitivity to steroids than γ-containing subtypes [13,27–29]. However, to date, relatively few steroids have been studied for effects on δ-containing $GABA_A$ receptors, so at present there is little information on whether these findings are true for all steroid types, and further, which molecular features of steroids contribute to receptor subtype-selectivity.

2. Materials and methods

2.1. Cell culture

Combinations of human $GABA_A$ receptor subunits were stably expressed in mouse L(tk−) cells to give α4β3δ and α4β3γ2 receptors [7,13]. The transfected cells were grown in Dulbecco's MEM with the addition of 10% Fetalclone II, and then maintained at 37 °C in 95% air and 5% CO_2. They were then plated into black-sided, clear bottom Porvair 96 well microtitre plates at a density of 0.8×10^4 cells/ml, with each well containing 200 μl medium. Experiments with these cells were carried out 24 h after induction of receptor expression with 1 μM dexamethasone as previously described [13].

2.2. Fluorescent measurement of membrane potential

L(tk−) cells were loaded with FRET dyes largely as previously described [13]. Briefly, cells were washed in a low Cl^- buffer which contained in mM: 160 sodium-gluconate, 4.5 potassium-gluconate, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 10 D-glucose, pH adjusted with NaOH to 7.4. This procedure was carried out using a PlateTrak™ (Packard Bioscience) leaving a 35 μl residual volume, followed by the addition of 65 μl of dye solutions containing the coumarin-linked phospholipid CC2-dimyristoyl phosphatidylethanolamine (CC2-DMPE) at a final concentration of 4 μM, together with the voltage-sensitive oxonol dye bis-(1,3-diethylthiobarbituric acid) trimethine oxonol ($DiSBAC_2(3)$) at a final concentration of 1 μM. Following a 30 min incubation period excess dye was removed with another buffer wash, and 55 μl of dye solutions containing $DiSBAC_2(3)$ and tartrazine ESS-Cy4 were added at final concentrations of 1 μM and 0.5 mM, respectively. The cell plates were then positioned into a Voltage/Ion Probe Reader (VIPR™, Aurora Biosciences Corporation, CA, USA) which simultaneously recorded changes in fluorescence at two emission wavelengths in response to addition of pharmacological stimuli. The Hamilton 2200 pipetter component of VIPR™ positions the microplate one row at a time over the fibre optic detection head, in this way light emission at two wavelengths (460 and 580 nm) is measured at 1 Hz from eight microtitre plate wells simultaneously. All the compound plates evaluated on VIPR had four wells containing dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) and an equal number of wells with propofol which were used as internal standard controls.

2.3. Data analysis

Background fluorescence from wells without L(tk−) cells in the microtiter plate was subtracted from that for each time point and for each fluorescence emission wavelength from other wells in the same plate. The ratiometric fluorescence at 460 nm to that at 580 nm was then calculated. This ratio became higher as the GABA-evoked depolarization increased. The automated calculations of fluorescence ratio and GABA responses was made using analysis templates written as Excel 97 macros (Microsoft corp.) [13,30]. Concentration response curves were fitted using standard 4 parameter fitting in 'Prism' (GraphPad Software Inc.). In the case of compounds which elicited both a potentiating and inhibitory effect, a sigmoidal curve was fitted to the rising portion of the curve to derive an EC_{50} and maximal efficacy but because of the biphasic nature of the curve the curves are illustrated with a connecting line. Where curves had not fully plateaued, a valid EC_{50} could not be accurately determined (ND).

2.4. Principal components analysis

Topologically based 2D atom-pair and topological torsion descriptors for the steroid compound set were generated using in-house 'topogen' software. The resulting 799 descriptors which were present in more than 10% of the data set were reduced, by means of an in-house principal components analysis software, to 10 orthogonal descriptors (principal components), which covered 94% of the variation within the 2D descriptor matrix [31]. A new data matrix was created which used the score values for each compound on these principal components as descriptors in conjunction with three other descriptors which encoded the 3D variation at atom positions 3, 5 and 20 in the steroid structure. Values of +1, 0, and −1 were assigned to beta, flat (i.e., "ene" or "-one"), or alpha stereochemical definitions at each of these three atomic centres. This 28 compound by 13 column data matrix was again reduced by means of principal components analysis, this time using SYBYL modelling software (TRIPOS Inc.) to three new principal components.

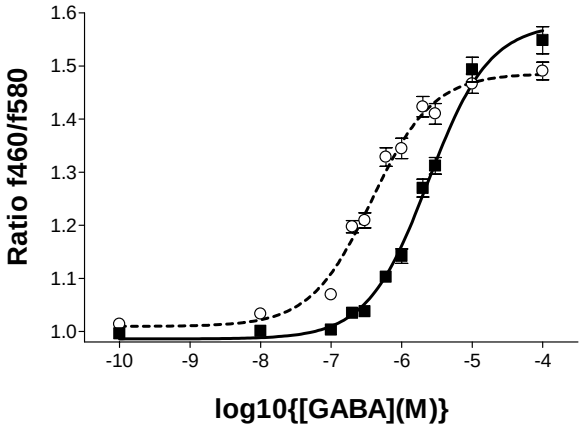


Fig. 1. Concentration response curves to GABA at $GABA_A$ α4β3δ and α4β3γ2 receptors. GABA was more potent at α4β3δ (open circles) $GABA_A$ than α4β3γ2 (closed squares) receptors: EC_{50} (mean ± S.E.M.) values of 0.40 ± 0.03 μM ($n = 18$) at α4β3δ and 2.41 ± 0.21 μM ($n = 17$) at α4β3γ2, respectively ($P < 0.0001$, unpaired two-tailed t test).

Table 1
The 28 steroid compounds have been arranged into three groups based upon the geometry at the 5-position

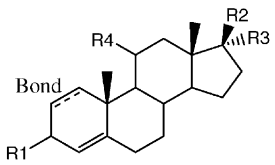
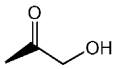
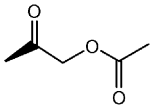
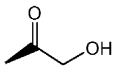
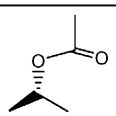
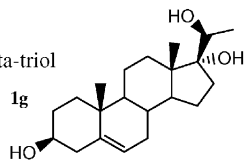
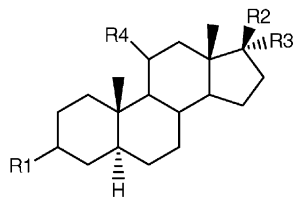
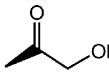
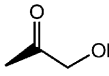
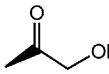
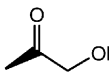
	R1	R2	R3	R4	Bond
4-pregnen-20-alpha-ol-3-one **1a**	=O		H	H	—
Prednisolone **1b**	=O		····OH	H	=
Prednisolone-21-acetate **1c**	=O		····OH	◀OH	=
Prednisone **1d**	=O		····OH	=O	=
4-pregnene-17alpha,20alpha-diol-3-one 20 acetate **1e**	=O		····OH	H	—
4-pregnene-17alpha,20-beta-diol-3-one **1f**	=O		····OH	H	—
5-pregnene-3beta,17alpha,20-beta-triol **1g**					

Table 1 (*Continued*)

	R1	R2	R3	R4
5-alpha-pregnane-3,11,20-trione **2a**	=O		H	=O
5-alpha-pregnane-3beta,11beta,17alpha,21-tetrol-20-one **2b**				
5-alpha-pregnane-3beta,21-diol-20-one **2c**			H	H
5-alpha-pregnane-3,20-dione **2d**	=O		H	H
5-alpha-pregnane-3beta-ol-20-one **2e**			H	H
5-alpha-pregnane-11beta,21-diol-3,20-dione **2f**	=O		H	
5-alpha-pregnane-3alpha,21-diol,20-one **2g**			H	H
5-alpha-pregnane-3alpha-ol,20-one **2h**			H	H
5-alpha-pregnane-3alpha,20alpha diol **2i**			H	H

2.5. Materials

CC2-DMPE was obtained from PanVera LLC and $DisBAC_2(3)$ from Cambridge Bioscience. Sodium D-gluconate, potassium D-gluconate, tartrazine, GABA, and $GABA_A$ receptor modulators were from Sigma. Magnesium chloride, calcium chloride, D-glucose and *N*-2-hydroxyethylpiperazine-*N*-2-ethansulphonic acid (HEPES) were obtained from BDH. Dulbecco's modified Eagle's medium was from Life Technologies, Inc., and Fetalclone II was from Hyclone (Logan, UT).

3. Results

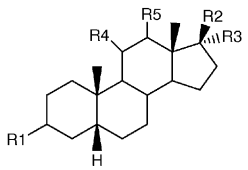
3.1. Agonist and standard modulator activity

In initial experiments, the effects of the agonist GABA, and known modulators of $GABA_A$ receptor function, were characterised in the inducible Ltk α4β3δ, and α4β3γ2, cell lines. A potentiometric assay based on fluorescence resonance energy transfer (FRET) was used to measure GABA-activated responses [13,32]. GABA was approximately six-fold more potent at the α4β3δ receptors compared to α4β3γ2 (Fig. 1), consistent with previous electrophysiological and fluorescence based findings from these cell lines [13,28]. With the benzodiazepine binding site ligand DMCM, there was inhibition of the agonist response at the α4β3γ2 receptors, whereas the effect of DMCM at α4β3δ was negligible (not shown). At both receptor subtypes there was marked potentiation by the anaesthetic propofol (not shown), as previously reported [13].

3.2. Steroid pharmacology

A structurally diverse set of steroids was selected for testing, to characterise differences in potency, efficacy, and selectivity at the steroid sites of α4β3δ and α4β3γ2 $GABA_A$ receptors. The number/letter code assigned to each steroid in Tables 1 and 2 is referred to when identifying a particular compound hereafter. Table 1 shows the structures for these steroids, and the potency and efficacy data of these compounds are summarized in Table 2. Most of the steroids evaluated in this study were found to

Table 1 (*Continued*)

	R1	R2	R3	R4	R5
5-beta-pregnane-3alpha,17alpha-diol-20-one **3a**	OH	O	OH	H	H
5-beta-pregnan-3beta-ol-20-one **3b**	OH	O	H	H	H
5-beta-pregnane-3alpha,20-beta-diol **3c**	OH	OH	H	H	H
5-beta-pregnane-3alpha,17alpha,20beta-triol **3d**	OH	OH	OH	H	H
5-beta-pregnane-3alpha,11beta,17alpha,20-beta-tetrol **3e**	OH	OH	OH	OH	H
5-beta-pregnane-3alpha,20alpha-diol diacetate **3f**	O O	O O	H	H	H
5-beta-pregnane-3alpha,17alpha,21-triol-20-one **3g**	OH	O OH	OH	H	H
5-beta-pregnane-3alpha,12 alpha-diol-20-one-diacetate **3h**	O O	O	H	H	O O
5-beta-pregnane-3alpha,17alpha-diol-11,20-dione **3i**	OH	O	OH	O	H
5-beta-pregnan-3alpha-ol-20-one **3j**	OH	O	H	H	H
5-beta-pregnane-11beta,17alpha, 21-triol-3,20-dione **3k**	O	O OH	OH	OH	H
5-beta-pregnane-3alpha,17alpha,20alpha-triol **3l**	OH	OH	OH	H	H

These are the 5-ene derivatives (the ene table includes the odd compound 5-pregnene, 3-β,-17α, 20 β-triol (**1g**) which has the double bond directed the opposite way to the other 5-ene compounds), the 5-alpha derivatives and the 5-beta derivatives. The compounds are defined by a number (1, 2 or 3) depending on which 5-position table they are in and then appended with a letter.

potentiate GABA responses at both subtypes. Concentration response curves for selected steroids in the presence of GABA EC_{50} are shown in Fig. 2. For most compounds there was a clear separation of their efficacy profiles between the receptor subtypes, with a substantially larger maximal response at the α4β3δ receptor. For example, large increases in GABA EC_{50} response potentiation were observed for (**3i**) (Fig. 2A) ($P = 0.005$, unpaired two-tailed *t* test), (**3b**) (Fig. 2B) ($P = 0001$) and (**2a**) (Fig. 2C) ($P = 0.03$) for the δ-containing subtype. Modulation of the GABA EC_{50} evoked by (**3c**) (Fig. 2D) was also highly subtype-selective, in this case with both greater efficacy ($P < 0.0001$) and potency at the δ-containing subtype. The maximum potentiation by this series of steroid compounds vary substantially but this did not appear to correlate closely with subtype selectivity. These findings are consistent with previously reported δ selectivity found for the steroids alfaxalone and tetrahydrodeoxycorticosterone [13,28,29,33]. The present study suggests that this is generally true across the class of steroids that can modulate GABA responses. Interestingly, however, there were several compounds in the present study which did not exhibit any significant selectivity between δ and γ2 subunits: (**3e**) had a more potent EC_{50} on α4β3δ compared to α4β3γ2, however, the maximum potentiation of the GABA responses was not significantly greater (Fig. 3A). Similarly, (**2d**) also does not appear to significantly differentiate between these two $GABA_A$ receptor subtypes (Fig. 3B).

From the large set of steroids assembled for this study, variability in the type of modulation, and the subtype

Table 2
Effects of steroid compounds on the EC_{50}GABA response at α4β3γ2 ($n = 6$ –15) and α4β3δ ($n = 8$–16) receptors

		α4β3γ2		α4β3δ	
		Max efficacy	EC_{50} (μM)	Max efficacy	EC_{50} (μM)
4-Pregnen-20α-ol-3-one	(**1a**)	−37	3.5	+31	0.07
Prednisolone	(**1b**)	No effect at 100 μM		+31	0.08
Prednisolone 21-acetate	(**1c**)	−28	15.6	33	0.26
Prednisone	(**1d**)	No effect at 100 μM		+42	0.07
4-Pregnene-17α,20α-diol-3-one-20-acetate	(**1e**)	−26	5.9	+22	0.5
4-Pregnene-17α,20β-diol-3-one	(**1f**)	No effect at 100 μM		+31	1.4
5-Pregnene-3β,17α,20β-triol	(**1g**)	No effect at 100 μM		+30	0.1
5α-Pregnane-3,11,20-trione	(**2a**)	+19	1.0	+82	2.2
5α-Pregnane-3β,11β,17α,21-tetrol-20-one	(**2b**)	No effect at 100 μM		+47	0.05
5α-Pregnane-3β,21-diol-20-one	(**2c**)	+17 at 100 μM		54	1.5
5α-Pregnane-3, 20-dione	(**2d**)	+20	0.08	+28	0.01
5α-Pregnan-3β-ol-20-one	(**2e**)	No effect at 100 μM		+47	0.07
5α-Pregnane-11β,21-diol-3,20-dione	(**2f**)	No effect at 100 μM		+75	10.0
5α-Pregnane-3α, 21-diol, 20-one	(**2g**)	+50	0.06	+220	0.09
5α-Pregnan-3α-ol-20-one	(**2h**)	+51	0.09	+140	0.09
5α-Pregnane-3α,20α-diol	(**2i**)	+111 at 100 μM	ND	+143 at 100 μM	13
5β-Pregnane-3α,17α-diol-20-one	(**3a**)	+20 at 100 μM	28	+57	3.5
5β-Pregnan-3β-ol-20-one	(**3b**)	+77	11.9	+243	9.1
5β-Pregnane-3α,20β-diol	(**3c**)	+85	23.3	+169	3.1
5β-Pregnane-3α,17α,20β-triol	(**3d**)	−16	7.9	+56	2.4
5β-Pregnane-3α,11β,17α,20β-tetrol	(**3e**)	+39	11.3	+29	2.7
5β-Pregnane-3α,20α-diol diacetate	(**3f**)	+28	ND	+64	2.3
5β-Pregnane-3α,17α,21-triol-20-one	(**3g**)	–23	18.7	+36	1.0
5β-Pregnane-3α,12α-diol-20-one-diacetate	(**3h**)	No effect at 100μM		+42 at 30 μM	0.3
5β-Pregnane-3α,17α-diol-11,20-dione	(**3i**)	+62	14.6	+134	10.7
5β-Pregnan-3α-ol-20-one	(**3j**)	+48	0.2	+280	0.4
5β-Pregnane-11β,17α,21-triol-3,20-dione	(**3k**)	+27 at 100μM		+73	12.0
5β-Pregnane-3α,17α,20α-triol	(**3l**)	+25	5.4	+55	1.5

Results are presented as maximal percent modulation of the EC_{50} GABA response based on curve fittings to the mean dataset. The number letter code in parentheses represents the structural grouping of compounds detailed in Table 1.

selectivity profile for different compounds is evident (Figs. 2–5). Several compounds were identified which inhibit GABA responses at α4β3γ2: (**3d**) (Fig. 4A), (**1c**), (**1e**), (**1a**) (Fig. 4B), and (**3g**) inhibited, although did not abolish, GABA responses at the subtype, while having modest-amplitude potentiation effects on α4β3δ. Sigmoidal 4 parameter curves in Figs. 2–4 were fitted to efficacy values at all the concentrations tested. Whereas previous studies have shown that pregnenolone sulphate can inhibit α4β3δ $GABA_A$ receptors [28], none of the non-sulphated steroid compounds evaluated in the present study decreased the α4β3δ GABA EC_{50} response except at very high concentrations, with the exception of (**2g**) (Fig. 5A).

The modulation of GABA EC_{50} by (**2g**) was unusual, in showing a biphasic response (Fig. 5A). At the α4β3δ receptor the peak positive modulation occurred at 0.3 μM with a response of 211.1 ± 17.7%, with the greatest negative modulation of −70.0 ± 8.9% taking place at 30 μM. At α4β3γ2, maximum potentiation of 58.4 ± 6.5% occurred at 1 μM, with maximal negative modulation of −56.6 ± 5.0% at 30 μM. Another distinct profile was seen with (**3j**) (Fig. 5B) which caused an increase in response with increasing concentration, followed by a decline after reaching the maximal peak response. This was seen at both receptor subtypes with the decline at α4β3δ being much greater. At α4β3δ the greatest response of 283.5 ± 32.2% occurred at 1 μM, at α4β3γ2 receptors 50.4 ± 9.0% potentiation was evident at 3 μM. For both Fig. 5A and B, responses are illustrated with a connecting line due to the nature of the biphasic effects; values quoted in Table 2 are derived from curve fitting to the rising portion of the concentration response curve.

The other steroids evaluated at these two receptor subtypes are shown in Table 2. Many of these compounds showed functional selectivity, in some cases accompanied by increased affinity at α4β3δ. Indeed all the steroid compounds evaluated displayed efficacy at the α4β3δ receptor. There were a number of compounds which did not have any discernible effect at α4β3γ2 (Table 2).

3.3. Kinetics of responses

The effects of steroids on the kinetics of GABA responses as measured by FRET was also evaluated in the present study. Examples of the effects of steroids on response kinetics are shown in Fig. 6. The normalized responses shown in Fig. 6A–D decrease at approximately 8 s due to an addition artifact associated with addition of

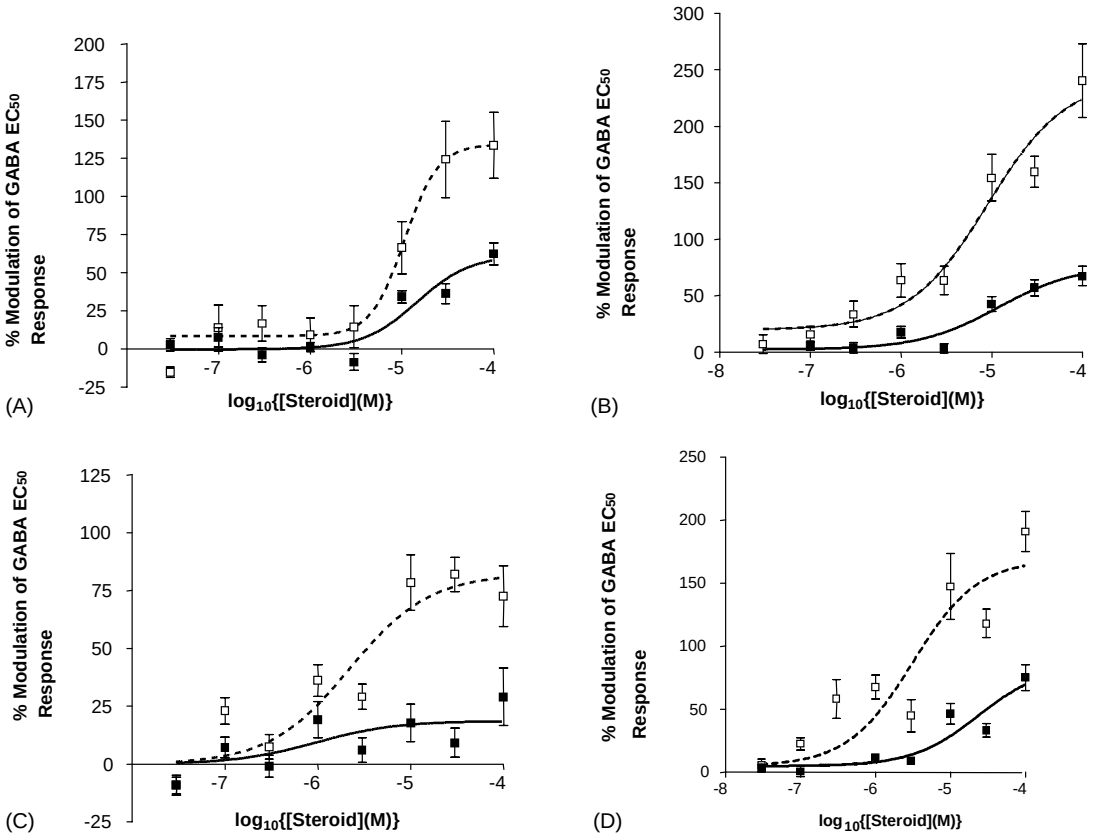


Fig. 2. Receptor subtype specific potentiation of EC_{50} GABA responses by steroids at α4β3δ (open symbols) and α4β3γ2 (closed symbols) receptors. (A) (**3i**), (B) (**3b**), (C) (**2a**), (D) (**3c**). Data shown are based on mean ± S.E.M. of seven to nine experiments.

buffer or steroid. The true membrane potential response occurs on addition of GABA, which evokes an increase in FRET and is subject to potentiation by test steroid. The time course plots for (**2g**) and (**1b**) show different kinetic profiles at the different receptor subtypes. The kinetics for (**2g**) shows a degree of apparent desensitization at α4β3γ2 receptors (Fig. 6B), which is not apparent at α4β3δ (Fig. 6A). In Fig. 6D, the GABA control shows some apparent desensitization (unlike the other controls), which may mask any effects that compound (**1b**) might be having on desensitization kinetics of α4β3γ2 receptors. Although some steroids have previously been shown by ourselves and others to display direct effects in activating $GABA_A$ receptors [13,28], this was not evident for the compounds evaluated in the present study.

3.4. Analysis of steroids subtype selectivity

Molecular modelling tools were utilised to examine the relationship between key structural features of this set of steroid compounds, and the level of their efficacy and selectivity for α4β3δ over α4β3γ2. A set of the 28 steroids evaluated was examined using principal components analysis on a data matrix of 2D atom-pair and topological torsion descriptors and three 3D indicator descriptors. Atom-pair descriptors cover the relationships between any non-hydrogen (heavy) atom and every other non-hydrogen atom in the molecule and are governed by the shortest, bonded path between the two atoms. Hence a typical atom-pair may be summarised in textual form by ‘‘a carbon 4 bonds away from an oxygen’’. More accurate descriptors can be made by defining the terminal atoms more precisely, e.g., ‘‘an aromatic carbon with three heavy atom connections being four bonds away from an oxygen in a hydroxyl group’’. Topological torsion descriptors cover groups of four heavy atoms which are bonded in a row of three bonds. A typical topological torsion descriptor could be expressed textually by ‘‘carbon bonded to carbon which is bonded to an oxygen which is bonded to a carbon’’ and as above each of the atoms listed in the descriptor can be given more accurate descriptions. Essentially the atom-pair descriptors encode global or long range properties of molecules whereas topological torsion descriptors encode small or local properties of molecules. The three 3D descriptors were included to take account of the epimeric variations at positions 3, 5 and 20 of the steroid which would not have been encoded by our in-house 2D descriptors. This is due to our descriptors being governed only by the shortest bonded path between atoms, and as such, they do not encode any information about direction or position in space of the atoms. With the set of steroids including both alpha and beta face derivatives, this information

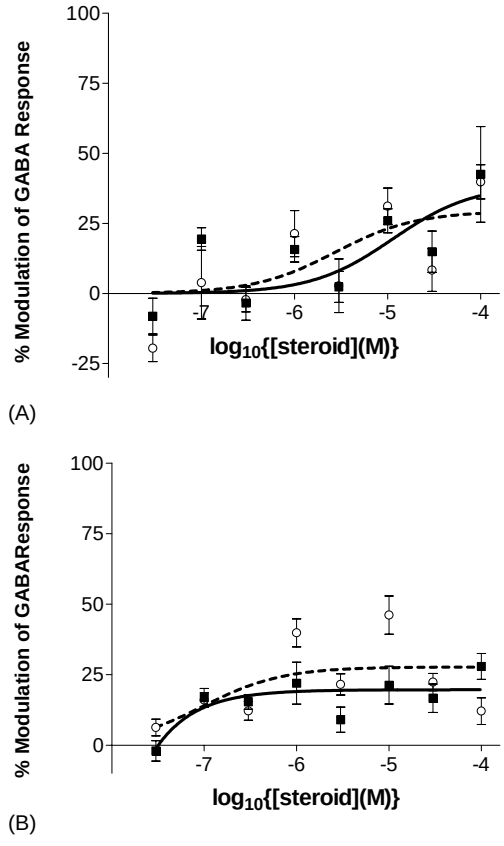


Fig. 3. Potentiation of EC_{50} GABA response by steroids at α4β3δ (open symbols) and α4β3γ2 (closed symbols) receptors not displaying receptor subtype-dependent maximum potentiation. (A) (**3e**), (B) (**2d**). Results are shown as mean ± S.E.M. and represent data from nine experiments.

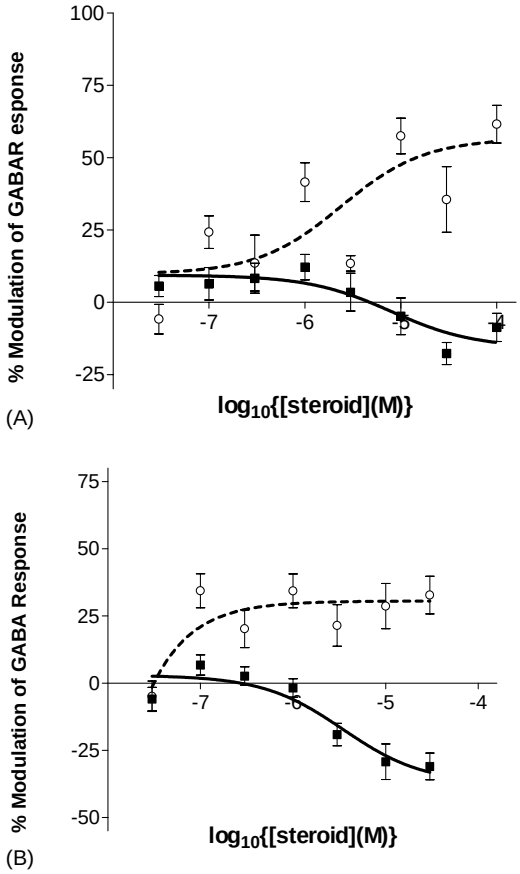


Fig. 4. Inhibition of the EC_{50} GABA response at α4β3γ2 (closed symbols) $GABA_A$ receptors by steroids.(A) (**3d**) and (B) (**1a**) reduced the amplitude of GABA responses at the α4β3γ2 subtype, while having modest-amplitude potentiating effects on α4β3δ (open symbols). Data shown are based on the mean ± S.E.M. of 8–15 experiments.

would be lost without the inclusion of another descriptor. The variations in direction at positions 3, 5 and 20 were described by allocating +1 for a β-face epimer, −1 for an α-face epimer and 0 if the position was involved in a double bond. Two of the data set structures are shown below and illustrate the α and β faces of the steroids (Fig. 7). Variations at the chiral centres of positions 3 and 5 could be linked if activity required some interaction close to the position of the 3-hydroxyl group, because 5β, 3β compounds and 5α, 3α compounds would place the hydroxyl group in the same position in space. The data matrix of molecules (rows) versus 2D descriptors (columns) was first submitted to a principal components analysis in order to reduce the number of descriptors from over 1000 to just only 10 independent descriptors. Principal components analysis does this by creating a combination descriptor which covers the maximum amount of variance within the data matrix. This first principal component is made up of contributions (or 'Loadings') from each of the original >1000 descriptors and the value for each molecule on this first principal component (or 'Scores') is the position of the molecule on the line which the first principal component represents. The next and subsequent principal components are created again by combination of the original descriptors, such that they are orthogonal to all the previous principal components and explain much of the residual variance of the data set once the previous principal components have been taken into account. In this way it is possible to take a very large data matrix (28 steroids versus >1000 descriptors) and transform it into a smaller one (28 steroids versus 10 principal components) while retaining the key information contained within the original. The 3D indicator variables mentioned above were then added to the above (28 × 10) data matrix to create a 28 × 13 data matrix which was subjected to a final principal components analysis.

The graphs produced show the Loadings (i.e., the contribution/importance of each descriptor to each principal component) and the Scores (i.e., the position of each molecule along each principal component) which were derived from this final principal components analysis. In the Loadings plot (Fig. 8), the first principal component has large and opposite influences from the epimeric nature at positions 3 and 5, whilst position 20 has no bearing on the first principal component but does have a major positive influence on the second principal component. Hence the epimeric preferences at these three positions largely

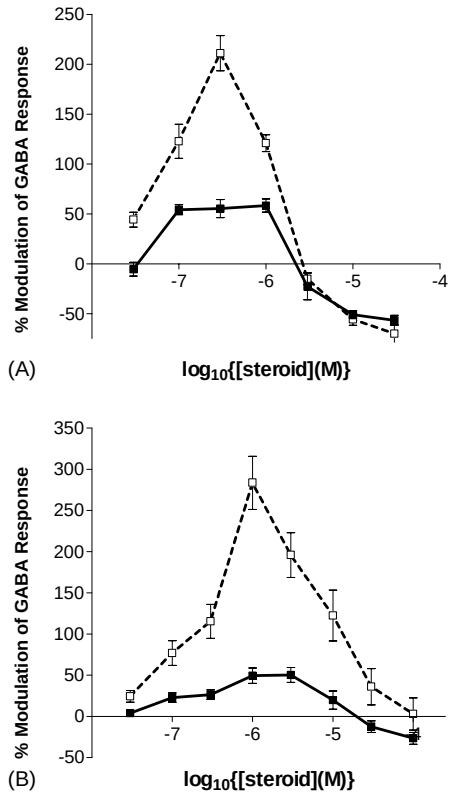


Fig. 5. Unusual biphasic modulation of EC_{50} GABA responses at α4β3γ2 (closed symbols) and α4β3δ (open symbols) receptors by (A) (**2g**), (B) (**3j**). Data shown are based on mean ± S.E.M. of 8–15 experiments.

explain the diversity in the steroid data set. If one examines the Scores plot (Fig. 9) then similar molecules should cluster in this plot. Highlighted in Fig. 9 are several compounds ((**3b**), (**3c**) and (**3i**) (Fig. 2)) which have high efficacy at α4β3δ, and clear selectivity in maximum efficacy over α4β3γ2. The fact that the three compounds with marked difference between their α4β3δ and α4β3γ2 efficacy are not together in this plot indicates that there are a number of reasons, not just one, for this selectivity. Of those molecules close to (**3b**) (i.e., (**1f**), (**1b**) and (**1c**)), all display lower maximum activity at α4β3δ than (**3b**), but retain selectivity, evoking virtually no potentiation at α4β3γ2, whilst (**1c**) partially inhibits the GABA response at α4β3γ2 receptors (Table 2). Close to (**3c**) are (**3d**) and (**3e**), the former retains functional selectivity for α4β3δ whereas the latter does not. Close to (**3i**) is only (**3l**) which again has modest selectivity but positive activity on both α4β3γ2 and α4β3δ, similar to (**3i**). Of the compounds which exhibit potentiation of α4β3δ and inhibition of α4β3γ2 (Fig. 4, Table 2), there is no co-localization within the Scores plot. However, it can be noted that the δ-selective potentiator (**2a**) (Table 2) is close in this plot to the δ-selective potentiator, and inhibitor (**2g**) (Fig. 5A). Less expectedly, there is a close localisation in the Scores plot for (**3c**), an efficacious and selective potentiator of α4β3δ (Fig. 2) with the non-selective, weak potentiator (**3e**) (Fig. 3A).

4. Discussion

In the present study, we have evaluated steroid modulation of two structurally related $GABA_A$ receptor subtypes which have been suggested to be pathologically linked to several types of seizure or epileptiform activities [25,34]. We have utilised a fluorescence based potentiometric assay which in recent years has become the standard industry tool for characterisation of voltage- and ligand-gated ion channel pharmacology [13,35,36]. This has facilitated an extensive study of the largest steroids set yet assembled for evaluation of molecular features within steroids which may account for subtype-selective effects on $GABA_A$ receptors containing the δ subunit. The responses to GABA at both receptor subtypes were robust and sizable, with GABA having an approximately six-fold greater potency at the α4β3δ receptor, in agreement with studies using whole-cell electrophysiological patch-clamp techniques [28,37].

The findings from this study may prove important in understanding the activity of endogenous neurosteroids, the levels of which, in addition to steroidogenic enzyme concentrations, will change in response to cyclic hormonal, chronic or acute episodes, e.g., menstrual cycle, pregnancy, stress or epilepsy. In a study where withdrawal of chronic exogenous neurosteroids was designed to mimic premenstrual syndrome in rats, levels of endogenous 3α-OH-5α-pregnan-20-one were reduced leading to increased seizure susceptibility through an enhancement of α4 gene transcription. Blockade of the α4 gene transcript prevented these withdrawal properties [22]. Several additional studies have since indicated that α4 and δ-containing receptors are upregulated within epilepsy, and implicate α4 and δ in the pathogenesis more specifically of temporal lobe epilepsy [25,34]. The use of δ subunit knockout mice using gene targetting technology, whereby there is specific disruption of genes by homologous recombination, provided initial evidence to the attenuated sensitivity of this receptor subtype to neurosteroids [24]. However, there are limitations to this knockout mutation strategy, which could result in adaptive changes during development of these animals [38].

The enhancement of the functional response to some neurosteroids in cell lines containing the δ subunit has recently been shown by imaging and electrophysiological studies [13,27,29]. In the present study, selectivity was found to be widespread amongst members of the steroid family tested, and was particularly marked with some compounds such as (**3b**), (**3c**) and (**3i**).

It has previously been reported that 4,5,6,7-tetrahydroisothiazolo-[5,4-c] pyridin-3-ol (THIP) is a 'super agonist' at α4β3δ containing receptors [28], this raises the possibility that GABA could be regarded as a partial agonist at this receptor, thus revealing greater potentiation of steroids at this sub-type. However, the results indicate a variety of efficacy profiles, with some steroids having opposite

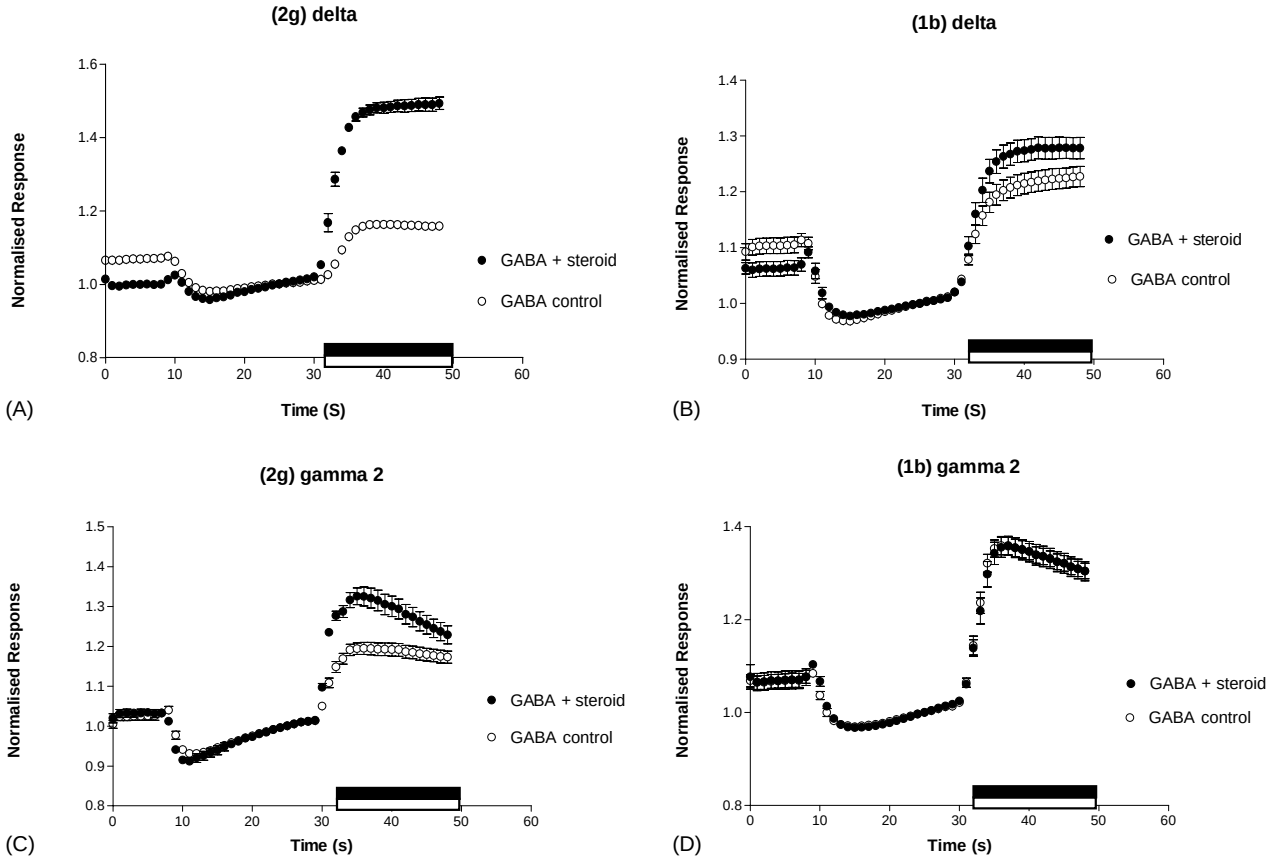


Fig. 6. Time course response for modulation of GABA EC_{50}response by 30 μM (**2g**) at (A) α4β3δ receptors and (B) α4β3γ2 receptors and 30 μM (**1b**) at (C) α4β3δ receptors and (D) α4β3γ2 receptors. The open bar shows the receptor stimulation with control EC_{50} GABA and the black bar shows the receptor stimulation with EC_{50} GABA in the presence of steroid. All data are the mean ± S.E.M. of eight experiments.

effects at delta and gamma2 receptors or potentiating the GABA response at both receptors.

The efficacy profile seen with (**2g**) was unusual in that it showed agonism at the lower concentrations tested, the activity being more marked at the δ receptor. At concentrations above 1 μM, there was antagonistic or even inverse agonist activity seen at both receptor subtypes, perhaps indicating an interaction with at least two different binding sites with this compound. A biphasic effect was seen with (**1a**), this compound appears to be a partial agonist at the delta containing receptor at nanomolar concentrations. At γ2 receptors, however, it is inactive or an antagonist at this concentration, while it is an inverse agonist at higher concentrations. This could again indicate the interaction with two different binding sites.

The focus of this investigation was to evaluate the set of steroids at these two $GABA_A$receptor subtypes, in particular to seek to understand the generality and structural

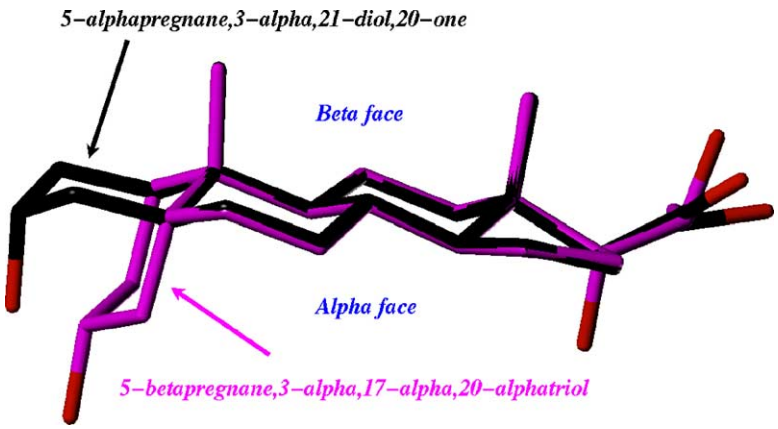


Fig. 7. Overlay of the steroids indicated to illustrate the effects of alpha or beta face epimeric form on the positions of key 3 and 5-position substituents.

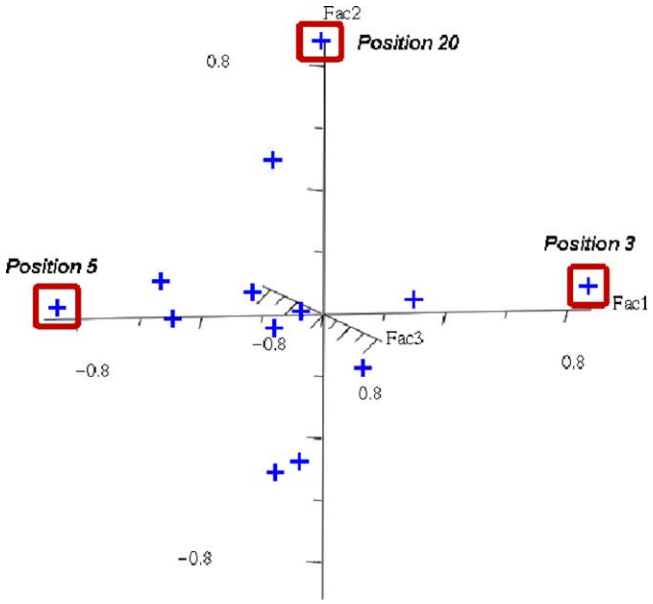


Fig. 8. Loadings plot from the principal components analysis of the complete data matrix of compounds and their structural descriptors.

basis of the preferential effect of steroids on δ-containing $GABA_A$ receptors which has previously been reported. The present results showed that most steroids showed greater modulation of the α4β3δ receptor. In virtually all cases for the non-sulphated compounds evaluated in this study, the modulation was a potentiation rather than an inhibition. A smaller number of compounds were found to be relatively non-selective between δ and γ2-containing subtypes, and another subset potentiated δ- while inhibiting γ2-containing receptor GABA responses. Molecular modelling was performed using principal components analysis, seeking structural motifs which might underlie these differential effects. This method has been applied in the present study to 2D and 3D structural descriptions of the steroids, so that structurally similar (and hopefully functionally similar) molecules would lie close to one another in the graphical representation of the principal components. However, there is concordance between functional selectivity and structural similarity for this compound set in only a limited set of cases. Several active and δ-selective potentiators, notably (**3b**), (**1f**), (**1b**) and (**1c**), are found in close proximity within the scores plot. However, we have also found compounds with very different functional profiles in close proximity within this plot, notably (**3c**) and (**3e**). Overall, the scores and loadings plots from this principal components analysis in Figs. 8 and 9 suggest that a number of potential structural sites are important for selectivity of interactions of steroids on $GABA_A$ receptors. A review article [39] suggests that steroids which have a positive modulation of GABAergic function have a 3α-OH group and a hydrogen bond accepting group on the β face of the steroid at the C-17 position. Another study, whereby conventional electrophysiological techniques were used to evaluate the structure activity relationships of analogues

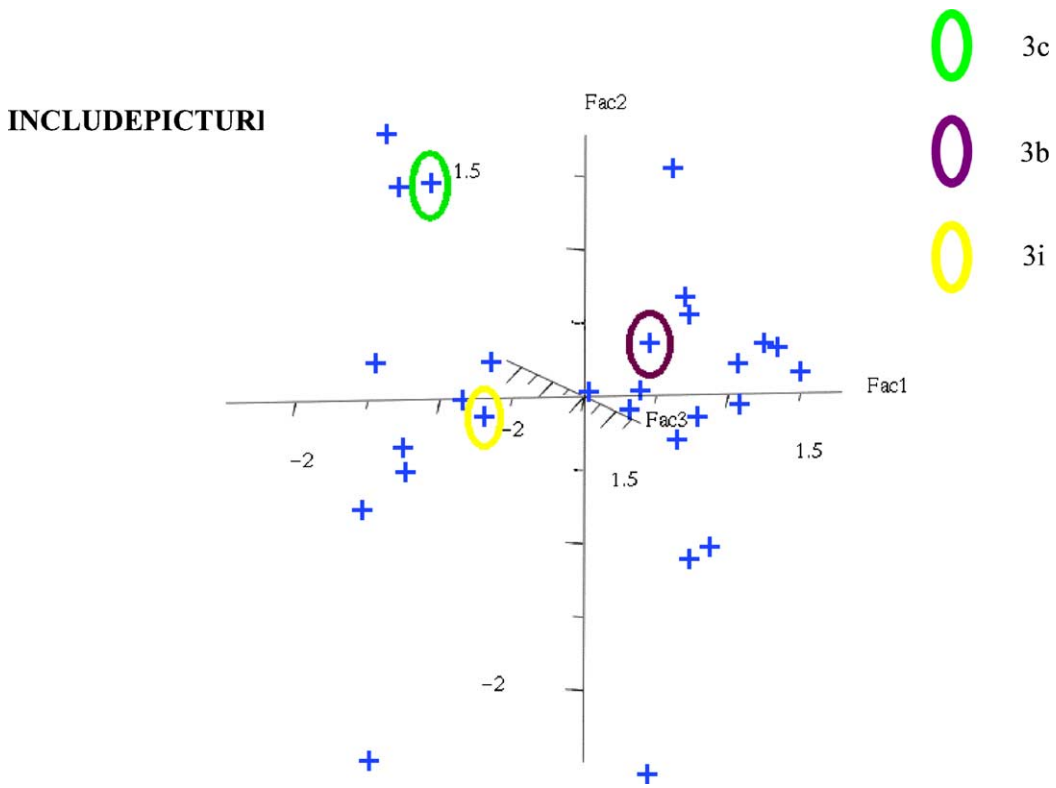


Fig. 9. Scores plot from the principal components analysis of the complete data matrix of compounds and their structural descriptors. Highlighted are selected compounds (**3c**), (**3b**) and (**3i**)) which have high efficacy at α4β3δ, and clear selectivity in maximum efficacy over α4β3γ2.

of steroid anaesthetics on rat $\alpha1\beta2\gamma2_L$GABA$_A$receptors expressed in *Xenopus laevis* oocytes, could not identify a compound which was markedly selective for this particular GABA$_A$receptor subtype [40].

It is clear from this study that there is not a single structural motif within steroid molecules which would account for the differential effects on GABA$_A$ receptor subtypes, a finding which may be consistent with steroid interactions at multiple sites on the GABA$_A$ receptor.

The steroid pharmacology of α4 and δ-containing GABA$_A$ receptors has been studied in greater detail in the present study than in any previous publication. We demonstrate that there is no simple correlation of steroid structure with efficacy at particular GABA$_A$ receptor subtypes. Rather, a complex picture emerges of multiple structural features underlying subtype-selective modulation of α4 and δ-containing GABA responses.

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