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RESEARCH PAPER

A 17β-derivative of allopregnanolone is a neurosteroid antagonist at a cerebellar subpopulation of $GABA_A$ receptors with nanomolar affinity

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Background and purpose: High-affinity, subtype-selective antagonists of the neurosteroid binding sites of $GABA_A$ receptors are not available. We have characterized an allopregnanolone derivative as an antagonist of cerebellar $GABA_A$ receptors with nanomolar affinity.
Experimental approach: Receptor binding and electrophysiological methods were used for the allosteric modulation of cerebellar $GABA_A$ receptors by an allopregnanolone derivative, (20R)-17β-(1-hydroxy-2,3-butadienyl)-5α-androstane-3α-ol (HBAO). $GABA_A$ receptors of rat cerebellar membranes were labelled with the chloride channel blocker [^{3}H]ethynylbicycloorthobenzoate (EBOB). The ionophore function of $GABA_A$ receptors was studied by whole-cell patch clamp electrophysiology in cultured rat cerebellar granule and cortical cells.
Key results: Partial displacement of cerebellar [^{3}H]EBOB binding by nanomolar HBAO was attenuated by 0.1 mM furosemide, an antagonist of α_6 and $\beta_{2\text{-}3}$ subunit-containing $GABA_A$ receptors. Displacement curves of HBAO were reshaped by 30 nM GABA and shifted to the right. However, the micromolar potency of full displacement by allopregnanolone was not affected by 0.1 mM furosemide or 30 nM GABA. The nanomolar, but not the micromolar phase of displacement of [^{3}H]EBOB binding by GABA was attenuated by 100 nM HBAO. Submicromolar HBAO did not affect [^{3}H]EBOB binding to cortical and hippocampal $GABA_A$ receptors. HBAO up to 1 μM did not affect chloride currents elicited by 0.3-10 μM GABA, while it abolished potentiation by 1 μM allopregnanolone with nanomolar potency in cerebellar but not in cortical cells. Furosemide attenuated cerebellar inhibition by 100 nM HBAO.
Conclusions and implications: HBAO is a selective antagonist of allopregnanolone, a major endogenous positive modulator via neurosteroid sites of cerebellar (probably $\alpha_6\beta_{2-3}\delta$) $GABA_A$ receptors.
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Keywords: $GABA_A$ receptor-ionophore; cerebellar granule cells; 17β-derivative of allopregnanolone; neurosteroid antagonist; furosemide; [^{3}H]EBOB binding; $\alpha_6\beta_{2\text{-}3}\delta$ $GABA_A$ receptors

Abbreviations: EBOB, ethynylbicycloorthobenzoate; HBAO, (20R)-17β-(1-hydroxy-2,3-butadienyl)-5α-androstane-3α-ol; K_A, dissociation constant; K_I, inhibition constant; TBOB, t-butylbicycloorthobenzoate; TBPS, t-butyl-bicyclophosphorothionate; THDOC, 5α-tetrahydrodeoxycorticosterone; TM, transmembrane

Introduction

GABA is the predominant inhibitory neurotransmitter in mammalian brains. Ionotropic $GABA_A$ receptors form membrane channels for the penetration of chloride and bicarbonate ions. Heteropentameric combinations of α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, $\rho_{1\text{-}3}$ and θ subunits lead to great structural and pharmacological variation in $GABA_A$ receptors (Whiting, 2003). The pharmacological fine-tuning of GABAergic neurotransmission can be performed by allosteric modulation via binding sites of $GABA_A$ receptors for benzodiazepines, barbiturates, anaesthetic alcohols and neurosteroids. Neurosteroids synthesized in the CNS are the only known endogenous allosteric modulators of $GABA_A$ receptors (Belelli and Lambert, 2005). Two major neurosteroids, allopregnanolone (5α-pregnan-3α-hydroxy-20-one) and its

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21-hydroxylated derivative 5α-tetrahydrodeoxycorticosterone (THDOC) (Figure 1) can be formed in the CNS from progesterone and deoxycorticosterone, respectively, both by 5α-reductase and 3α-hydroxysteroid dehydrogenase (Agis-Balboa *et al*., 2006). Fluctuations of nanomolar allopregnanolone and THDOC have been considered to modulate $GABA_A$ receptors during the ovarian cycle, pregnancy (Concas *et al*., 1998), stress, prenatal (Bailey *et al*., 1999) and postnatal development (Mtchedlishvili *et al*., 2003) and aging (Schumacher *et al*., 2003) both directly and by upregulation of δ subunit-containing $GABA_A$ receptors (Maguire and Mody, 2007). Neurosteroids have multiple, stereoselective effects on $GABA_A$ receptors. Nanomolar concentrations of these 3α-hydroxy-pregnanes potentiate (Majewska *et al*., 1986) and micromolar concentrations directly activate $GABA_A$ receptor ionophores, while micromolar concentrations of the sulphates of 3β-hydroxy-pregnanes such as pregnenolone sulphate inhibit them (Park-Chung *et al*., 1999). The potencies and efficacies of neurosteroids depend on the subunit composition of $GABA_A$ receptors (Belelli *et al*., 2002; Rahman *et al*., 2006). The replacement of γ_2 subunits with δ and ε subunits leads to higher affinity for agonists and neurosteroids, especially in extrasynaptic $\alpha_6\beta\delta$, $\alpha_4\beta\delta$ and constitutively open $\alpha_1\beta_3\varepsilon$ $GABA_A$ receptors (Adkins *et al*., 2001; Wohlfarth *et al*., 2002; Bianchi and Macdonald, 2003; Maksay *et al*., 2003).

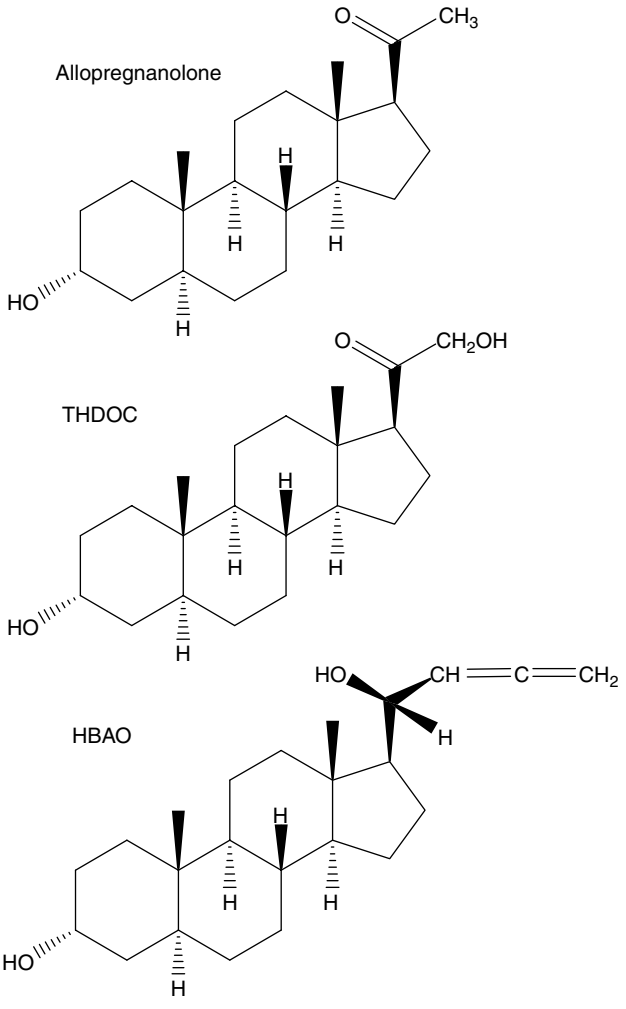

Figure 1 Chemical structures of HBAO, allopregnanolone and 5α-THDOC.

Binding of radiolabelled cage convulsants such as [^{35}S]t-butyl-bicyclophosphorothionate (TBPS) and [^{3}H]t-butylbicycloorthobenzoate (TBOB) to their binding sites within the anion channels of $GABA_A$ receptors (Perret *et al*., 1999) has been useful to detect functionally relevant allosteric interactions (Maksay, 1996). [^{35}S]TBPS binding has revealed the allosteric modulation of $GABA_A$ receptors via binding sites of neurosteroids (Gee *et al*., 1988) and the heterogeneity of neurosteroid binding (Hawkinson *et al*., 1994). [^{3}H]EBOB is another cage convulsant having higher affinity than [^{3}H]TBOB and a radiolabel of substantially longer half-life than [^{35}S]TBPS (Cole and Casida, 1992). [^{3}H]EBOB has enabled us to demonstrate nanomolar binding of agonists and neurosteroids to cerebellar $GABA_A$ receptors (Maksay and Bíró, 2005). This has been associated with $\alpha_6\beta_{2\text{-}3}\delta$ $GABA_A$ receptors in cerebellar granule cells (Fodor *et al*., 2005).

Medicinal chemists have frequently modified allopregnanolone, the pivotal GABAergic neurosteroid: substitution in position 11 has led to the surgical anaesthetic alphaxalone (Lambert *et al*., 1987), while 3β-alkyl substituents to the antiepileptic ganaxolone and the hypnotic CCD3693 (Carter *et al*., 1997; Gasior *et al*., 1999). The therapeutic benefits of all these 3α-hydroxy derivatives originate from the positive modulation of $GABA_A$ receptors. Opposite chirality of 5β-reduced and 3β-hydroxy-pregnanes and especially their sulphate esters decrease and invert the efficacies of neuroactive steroids. Thus, 5β-THDOC is a partial agonist (Xue *et al*., 1997), while sulphate esters of pregnenolone and dehydroepiandrosterone inhibit the ionophore activity of $GABA_A$ receptors (Park-Chung *et al*., 1999). (3α,5α)-17-Phenyl-androst-16-en-3-ol is a selective antagonist of 5α-reduced neurosteroids (Mennerick *et al*., 2004). Extensive studies have explored the structure–activity relationships of neuroactive steroids and revealed the requirement of a hydrogen bond accepting group in 17β position (Covey *et al*., 2001). The 17β unsaturated derivative of allopregnanolone, (20R)-17β-(1-hydroxy-2,3-butadienyl)-5α-androstane-3α-ol (HBAO) (Figure 1) has shown outstanding potency and stereoselectivity to modulate a cerebellar subpopulation of $GABA_A$ receptors (Souli *et al*., 2005). Here, we demonstrate that this can be attributed to antagonism of allopregnanolone potentiation of a high-affinity population of probably $\alpha_6\beta_{2\text{-}3}\delta$ $GABA_A$ receptors with nanomolar potency in cerebellar granule but not in cortical cells.

Materials and methods

Membrane preparation and [^{3}H]EBOB binding

The experimental protocols were approved by the Veterinary and Food Control Station of Budapest, in agreement with the guidelines of the European Communities Council Directive (86-609-EEC). Male Wistar rats were decapitated; brain regions dissected, homogenized in 0.32 M sucrose and centrifuged at 1000 *g* for 10 min. The supernatant was centrifuged at 45 000 *g* for 30 min. Pellets were homogenized

in distilled water, centrifuged at 45 000 *g* for 30 min, washed by suspension in 50 mM Tris–HCl buffer (pH 7.4) and similar centrifugations twice and frozen. The thawed suspensions were centrifuged in 50 mM Tris–HCl containing 0.2 M NaCl at 10 000 g for 10 min and washed by a similar centrifugation.

For displacement studies, triplicate membrane suspensions were incubated with 1 nM [^{3}H]EBOB, allopregnanolone and GABA for 2 h at 25°C. For nonspecific binding, 50 μM picrotoxinin was applied. Samples of 1 ml were filtered on Whatman GF/B filters under vacuum with a Brandel Harvester. Radioactivity was measured by scintillation spectrometry.

Cell culturing

Primary cerebellar cultures were prepared from 4-day-old Wistar rats and cortical cultures from 17-day-old rat embryos. After decapitation, cerebellar and cortical tissues were dissected, washed with Ca^{2+}–Mg^{2+}-free HEPES-buffered solution (in mM: NaCl 137, KCl 5, $NaHCO_3$ 3, Na_2HPO_4 0.6, KH_2PO_4 0.4, *d*-glucose 5.6, HEPES 20, penicillin G 100 U ml^{-1}, streptomycin 100 mg l^{-1}, amphotericin B 0.25 μg ml^{-1}, pH 7.4), and incubated in 1 × trypsin–EDTA solution for 3 min. After trituration, cell suspensions were filtered through a 70 μm mesh and centrifuged at 125 *g* twice for 5 min. Pellets were resuspended in culture medium (DMEM), supplemented with 10% fetal bovine serum, nerve growth factor 20 ng ml^{-1}, KCl 20 mM, amphotericin B 0.25 μg ml^{-1}, penicillin G 100 U ml^{-1} and streptomycin 100 μg ml^{-1}. Cells were plated at densities of 1–1.5 × 10^5 cells cm^{-2} on sterilized glass cover slips coated with poly-*d*-lysine. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2–95% air; half of the medium was changed to fresh serum-free medium twice a week. Primary cortical cultures were similarly prepared.

Patch-clamp electrophysiology

Conventional whole-cell patch-clamp recordings were made from cultured cerebellar and cortical cells 7–20 days after plating. Cultures in a recording chamber were superfused with the extracellular solution (e.s.) at 25°C. The e.s. contained (in mM): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, HEPES 5, HEPES-Na 5, glucose 20, pH 7.35. Patch electrodes (resistances: 4–7 MΩ) pulled from borosilicate capillary glass were filled with the intracellular solution (i.s.). The composition of i.s. was (in mM): CsCl 110, $MgCl_2$ 4.5, HEPES 9, BAPTA 10, ATP-Na_2 4, GTP 0.3, creatine phosphate 14, creatine phosphokinase 50 U ml^{-1}, pH 7.25. Osmolarities of the e.s. and i.s. were 310 and 290 mOsm, respectively. Tetrodotoxin (0.3 μM in the e.s.) was used to block action potentials. The compounds diluted in the e.s. (0.2% DMSO) were applied onto the cells via multi-barrelled, pressure-driven ejection pipettes controlled by electromagnetic valves. GABA was applied repetitively for 3 s at 30-s intervals with or without 1 μM allopregnanolone, HBAO and 100 μM furosemide. Inward currents were low-pass filtered (1 kHz) and recorded at a holding potential of −70 mV using an Axopatch 200A amplifier, digitized (Digidata 1200), captured and analysed using pClamp 8.0 (Axon Instruments, Union City, CA, USA).

Data evaluation

Curve fitting was performed via GraphPad Prism 4.02. (San Diego, CA, USA). Displacement of [^{3}H]EBOB binding by GABA was fitted with two sites. Where displacement phases were small, fitting was applied to the pooled experimental data. Biphasic displacement by HBAO plus GABA was fitted with a bell-shaped curve. Since fitting is based on logarithmic concentrations, IC_{50} values are shown with 95% confidence intervals instead of their asymmetric standard deviations.

In electrophysiology, baseline currents (current values before agonist application) were subtracted from the peak amplitudes of inward currents evoked by GABA. Potentiation was calculated from the comparison of the peak currents in the presence and absence of allopregnanolone. Concentration-dependent effects of HBAO were evaluated from the inhibition of current amplitude enhancements by allopregnanolone as the difference between the current amplitudes with and without allopregnanolone. Pooled data were fitted with a sigmoid curve and variable slope yielding the inhibitory IC_{50} value. The inhibition constant (K_I) of HBAO was calculated from its IC_{50} value according to the Cheng–Prusoff equation

$$K_I = \frac{IC_{50}}{1 + [A]/K_A}$$

where [A] is the concentration of allopregnanolone and its dissociation constant $K_A = 14$ nM as determined before (Fodor *et al*., 2005).

Materials

[^{3}H]EBOB (30 Ci mmol^{-1}) was purchased from Dupont-NEN and freshly diluted for the experiments. HBAO was synthesized as described (Souli *et al*., 2005). Allopregnanolone was purchased from Steraloids Inc. (Newport, USA); culture medium (DMEM) supplemented with 10% fetal bovine serum from Gibco (Gaithersburg, MD, USA); tetrodotoxin from Latoxan (Valence, France); borosilicate capillary glass (GC120F-10) from Harvard Apparatus (Edenbridge, UK). Other drugs and reagents (furosemide, penicillin, streptomycin, trypsin, poly-D-lysine, nerve growth factor and amphotericin) were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). HBAO and allopregnanolone were dissolved in DMSO the final concentration of which did not exceed 0.3% in membrane suspensions and cell cultures. Aqueous concentrations of HBAO can be considered as reliable up to its solubility limit of about 3 μM.

Results

GABA shifted and furosemide attenuated the displacement of cerebellar [^{3}H]EBOB binding by HBAO but not allopregnanolone

Concentration-dependent effects of HBAO and allopregnanolone were studied on [^{3}H]EBOB binding to rat cerebellar $GABA_A$ receptors. HBAO displaced [^{3}H]EBOB binding partially (Figure 2) and with nanomolar potency as reported

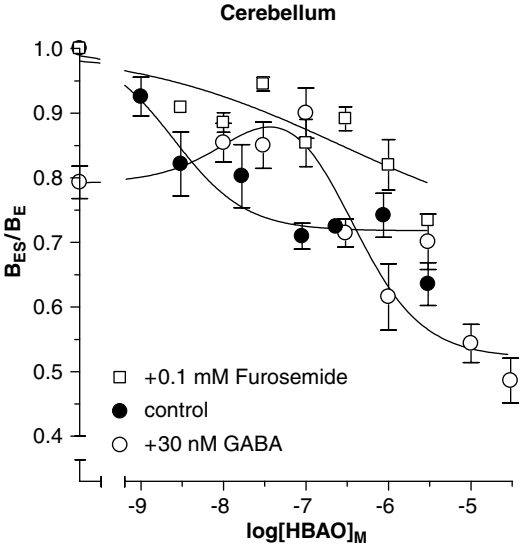

Figure 2 Concentration-dependent displacement of [^{3}H]EBOB binding to rat cerebellar $GABA_A$ receptors by HBAO in the absence and presence of 0.1 mM furosemide and 30 nM GABA. Ratios of [^{3}H]EBOB binding in the presence of the steroidal HBAO (B_{ES}) and its absence (B_E). Note that displacements in the presence of furosemide are related to the intrinsic enhancing effects of 0.1 mM furosemide. Control HBAO data are the extension of those in Souli *et al.* (2005). Points are means ± s.e.m., $n = 3$–7.

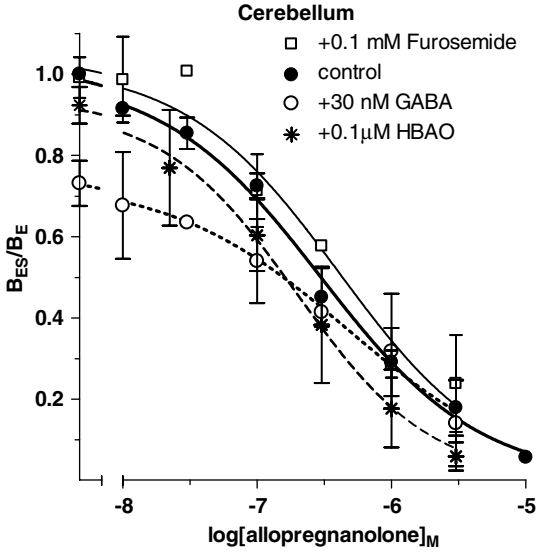

Figure 3 Concentration-dependent displacement of [^{3}H]EBOB binding to rat cerebellar $GABA_A$ receptors by allopregnanolone, in the presence of 0.1 mM furosemide, 30 nM GABA (dotted line) and 100 nM HBAO (dashed line). Points are means ± s.e.m., $n = 3$–6.

(Souli *et al.*, 2005). Sigmoid fitting to the pooled data of six experiments resulted in $IC_{50} = 2.3$ (1.3–3.9) nM (95% confidence interval). Figure 2 also shows that 30 nM GABA displaced [^{3}H]EBOB binding to 79.0 ± 0.3% (mean ± s.e.m.) and resulted in a biphasic displacement curve of HBAO shifted to the right. Fitting a bell-shaped curve resulted in $IC_{50} = 181$ (11–2950) nM (seven pooled experiments). The antagonist of $GABA_A$ receptors containing α_6 and β_{2-3} subunits (Korpi *et al.*, 1995), furosemide at 0.1 mM increased cerebellar [^{3}H]EBOB binding slightly, to 109.0 ± 3.0% (mean ± s.e.m. of three experiments). More importantly, furosemide strongly attenuated displacement by HBAO (Figure 2). When concentration-dependent displacements were normalized to the enhancement by 0.1 mM furosemide (Figure 2), sigmoid curve fitting resulted in a displacing potency of about two orders of magnitude weaker ($IC_{50} \sim 0.3\ \mu M$) than without furosemide.

Displacement by allopregnanolone was examined for comparison. It resulted in full displacement of specific [^{3}H]EBOB binding to cerebellar $GABA_A$ receptors (Figure 3) with $IC_{50} = 299$ (208-426) nM and slope values of 0.74 ± 0.09 (means of four experiments). The presence of 30 nM GABA and 0.1 mM furosemide did not significantly affect the displacing potencies of allopregnanolone (Figure 3): $IC_{50} =$ 478 (144–1580) nM for GABA and $IC_{50} = 406$ (204–812) nM for furosemide. It should be noted, however, that a higher concentration (400 nM) of GABA increased the displacing potency of allopregnanolone by about one order of magnitude (Fodor *et al.*, 2005). Finally, the presence of 100 nM HBAO slightly shifted the displacement curve of allopregnanolone in Figure 3 but again the potency was not significantly affected: $IC_{50} = 196$ (70-537) nM.

Nanomolar displacing effects of HBAO were restricted to cerebellar [^{3}H]EBOB binding. Figure 4 shows that HBAO, up to 1 μM, did not significantly affect [^{3}H]EBOB binding to cortical and hippocampal $GABA_A$ receptors.

HBAO attenuated only the nanomolar phase of [^{3}H]EBOB displacement by GABA

Displacement of cerebellar [^{3}H]EBOB binding by GABA was biphasic, as characterized by nanomolar and micromolar phases (Maksay and Bíró, 2005). Figure 5 shows that 100 nM HBAO selectively attenuated the nanomolar phase of displacement: Fitting two-phasic displacement curves to the pooled data of seven control experiments resulted in a high-affinity fraction of 34 ± 5% with $IC_{50,1} = 3.2$ (1.2–8.3) nM and a lower affinity of $IC_{50,2} = 0.8$ (0.5–1.4) μM. The presence of 100 nM HBAO halved the high-affinity fraction to 16.0 ± 2.8%, while the displacing potencies were not affected: $IC_{50,1} = 3.0$ (0.7–12.4) nM and $IC_{50,2} = 0.6$ (0.4–0.7) μM (three pooled experiments).

HBAO antagonized the enhancement by allopregnanolone of GABA-elicited chloride currents in cerebellar granule, but not in cortical cells, and furosemide attenuated this antagonism

Chloride currents were measured via patch-clamp electrophysiology in cultured cerebellar granule cells. Table 1 shows that chloride currents by 0.3–10 μM GABA were not affected significantly by HBAO up to 1 μM. Allopregnanolone elicited polyphasic enhancements of the chloride currents (Fodor *et al.*, 2005). Allopregnanolone enhancement was studied at 1 μM, a maximally potentiating concentration, and with 1 μM

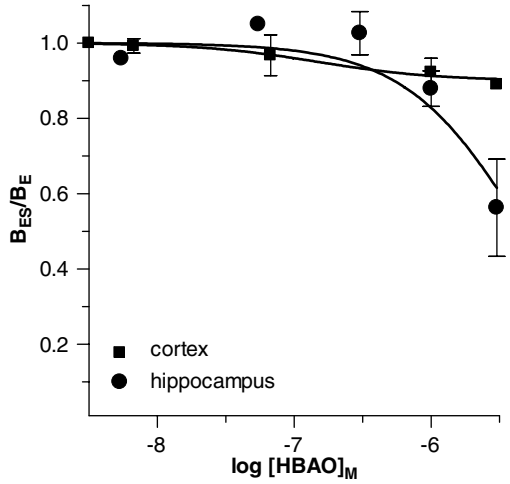

Figure 4 Slight displacing effects of HBAO on [^{3}H]EBOB binding to cortical and hippocampal $GABA_A$ receptors. Points are means$\pm$ s.e.m., $n=3$.

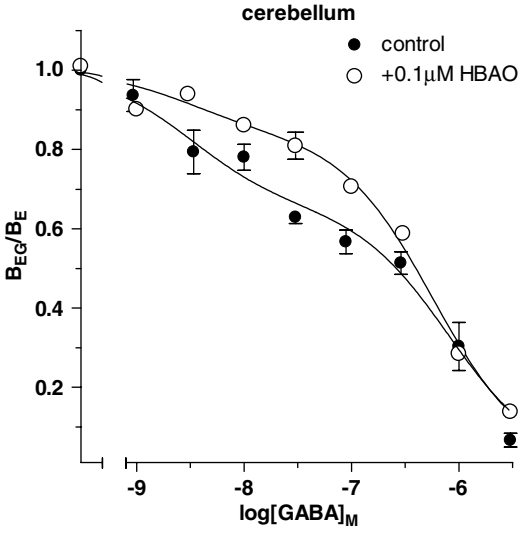

Figure 5 Heterogeneous displacement of cerebellar [^{3}H]EBOB binding by GABA (means$\pm$s.e.m., $n=7$) and the effects of 100 nM HBAO ($n=3$).

GABA. It resulted in current enhancements of $141\pm28\%$ (mean$\pm$s.e.m. of 21 experiments). Figure 6a demonstrates representative traces showing that HBAO antagonized the enhancement by allopregnanolone. HBAO exerted concentration-dependent, complete antagonism of the enhancement by allopregnanolone as shown in Figure 7. Sigmoid curve fitting resulted in $IC_{50}=35$ (23–53) nM and a slope value of 1.33 ± 0.43 (mean$\pm$s.e.m.). The IC_{50} value was converted into $K_I=0.49$ nM HBAO. Current peaks elicited by 1 μM GABA were about half the size in the presence of 0.1 mM furosemide as described before (Fodor *et al.*, 2005). Allopregnanolone (1 μM) enhanced the GABA-elicited chloride currents in the presence of 0.1 mM furosemide less by $51\pm11\%$ (mean$\pm$s.e.m. of eight experiments). Furosemide attenuated not only the enhancement by allopregnanolone

Table 1 HBAO did not significantly affect the GABA-elicited chloride currents in cerebellar granule cells

HBAO (nM)	*GABA (μM)*	*Current (%)*	*s.e. mean*	n
10	1	96.4	4.1	7
100	1	100.3	9.0	6
1000	1	81.6	7.5	6
100	0.3	106.7	7.5	6
100	3	96.1	1.8	6
100	10	97.3	0.9	5

Chloride current peaks elicited by GABA in the presence of HBAO were expressed in percent of control (in the absence of HBAO). Data are mean$\pm$s.e.m. of n experiments. They were not different significantly ($P>0.05$) from control in Student's paired t-test.

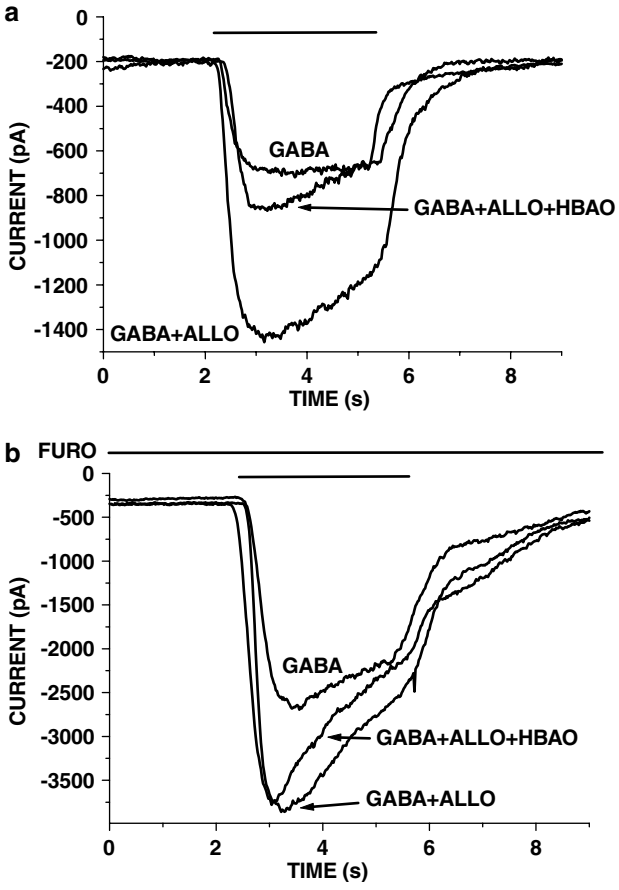

Figure 6 Representative electrophysiological recordings show the effects of HBAO on allopregnanolone-enhanced GABA currents in cerebellar granule cells in the absence (**a**) and in the continuous presence (**b**) of 0.1 mM furosemide. GABA, allopregnanolone and HBAO were applied for 3 sec indicated by the short horizontal bar. Overlapping traces represent currents evoked by 1 μM GABA in the absence and presence of 1 μM allopregnanolone and 100 nM HBAO.

but also its inhibition by 100 nM HBAO as shown on the representative traces in Figure 6b and summarized in Table 2.

For comparison, the effects of HBAO were also studied in primary cortical cultures in identical conditions. Allopregnanolone (1 μM) enhanced the chloride currents elicited

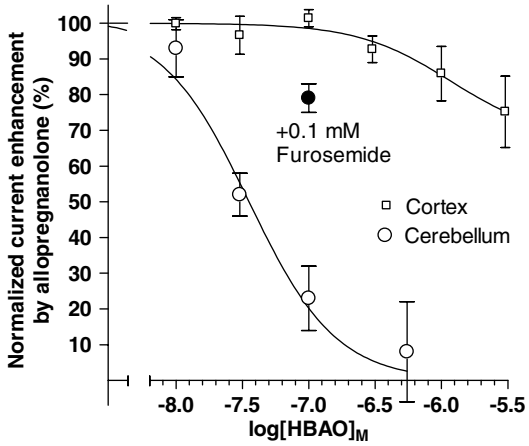

Figure 7 The effects of HBAO on the enhancements by 1 μM allopregnanolone of the chloride currents elicited by 1 μM GABA in cultured rat cerebellar granule and cortical cells. Cerebellar and cortical GABA-elicited chloride current peaks were enhanced by 1 μM allopregnanolone by 141 $\pm$ 28% (mean $\pm$ s.e.m., $n = 21$) and by 47 $\pm$ 9% ($n = 22$), respectively. Data are % current enhancements by allopregnanolone in the presence of different concentrations of HBAO (means $\pm$ s.e.m., $n = 4$–12). Cortical inhibition did not reach statistical significance. Cerebellar inhibition by 100 nM HBAO attenuated in the presence of 0.1 mM furosemide was also plotted for comparison.

Table 2 Furosemide attenuated the inhibition by HBAO of the enhancements by allopregnanolone of $GABA_A$ currents in rat cerebellar granule cells

GABAergic agents	*% of enhancement*
1 μM GABA + 1 μM allopregnanolone + 0.1 μM HBAO ($n = 6$)	23.0 $\pm$ 8.6
0.1 mM furosemide + 1 μM GABA + 1 μM allopregnanolone + 0.1 μM HBAO ($n = 8$)	79.1 $\pm$ 4.3*

Means $\pm$ s.e.m. of n experiments indicated in parentheses. Data are expressed in percent of the current amplitude enhancement by allopregnanolone. Currents were evoked by 1 μM GABA in the absence (first line) or in the continuous presence (second line) of 0.1 mM furosemide.
*$P < 0.0001$, significantly different from control (without furosemide); Student's *t*-test.

by 1 μM GABA by 47 $\pm$ 9% (mean $\pm$ s.e.m., $n = 22$). Figure 7 shows that no effects were observed up to 0.1 μM HBAO followed by slight, concentration-dependent inhibition of the enhancement.

Discussion

This study demonstrated selective binding and functional antagonism of a cerebellar subpopulation of $GABA_A$ receptors by HBAO. Submicromolar HBAO antagonized a nanomolar component of displacement of [^{3}H]EBOB binding by GABA. Moreover, HBAO antagonized the potentiation by allopregnanolone of $GABA_A$ receptor ionophores of cerebellar granule cells with nanomolar potency. High occupancy of the neurosteroid sites by allopregnanolone did not enable us to determine exactly the K_I value of HBAO. However, the calculated K_I value of 0.49 nM is not far from the dissociation constant of HBAO (2.8 nM) determined from allosteric displacement of cerebellar [^{3}H]EBOB binding (Souli *et al.*, 2005). The relationship is not clear between HBAO antagonism of nanomolar GABA displacement and that of allopregnanolone potentiation of micromolar GABA function. However, HBAO has rather unprecedented high affinity as an antagonist of neurosteroids potentiating $GABA_A$ receptors.

High-affinity cerebellar $GABA_A$ receptors: $\alpha_6\beta_{2/3}\delta$ subunit combination?

The displacement of cerebellar [^{3}H]EBOB binding has recently revealed high-affinity binding of agonists (nanomolar for GABA and micromolar for taurine); nanomolar binding of THDOC and allopregnanolone; and strong positive cooperativity between these neurosteroids and $GABA_A$ agonists (Maksay and Bíró, 2005). Further, furosemide, a selective antagonist of $GABA_A$ receptors containing α_6 and $\beta_{2\text{-}3}$ subunits (Korpi *et al.*, 1995) eliminated only the nanomolar displacing phase of GABA (Maksay and Bíró, 2005) and the potentiation by nanomolar allopregnanolone of GABA-elicited chloride currents in cerebellar granule cells (Fodor *et al.*, 2005). These findings have been correlated with the contribution of $\alpha_6\beta_{2\text{-}3}\delta$ $GABA_A$ receptors in granule cells.

Allopregnanolone has not shown any significant regional or subtype selectivity in displacement of the other cage convulsant [^{35}S]TBPS to $GABA_A$ receptors (Hawkinson *et al.*, 1994). Similarly, the displacing potency of allopregnanolone did not appear to differ for [^{3}H]EBOB binding to different $GABA_A$ receptors in cerebellum and the slope factor of allopregnanolone displacement was close to unity here. Consequently, furosemide blockade of $GABA_A$ receptors containing α_6 subunits did not shift the potency of allopregnanolone. Further, 30 nM GABA only displaced the high-affinity $GABA_A$ receptors but it did not affect the potency of allopregnanolone for the others. In contrast, selective binding of HBAO to a subpopulation of cerebellar $GABA_A$ receptors was strongly affected by 30 nM GABA and furosemide.

The involvement of $\alpha_6\beta\delta$ $GABA_A$ receptors in HBAO antagonism was supported by the following six findings: (1) HBAO eliminated the nanomolar phase of cerebellar [^{3}H]EBOB displacement by GABA; (2) furosemide attenuated the nanomolar displacement by HBAO; (3) HBAO antagonized the enhancement by allopregnanolone of GABA-elicited chloride currents in granule cells; (4) furosemide attenuated this enhancement by allopregnanolone; (5) furosemide also attenuated the inhibition by HBAO of the allopregnanolone enhancement; and (6) HBAO up to 1 μM was ineffective on cortical and hippocampal [^{3}H]EBOB binding and on allopregnanolone enhancement of GABA-elicited chloride currents in cortex. Points 4 and 5 can be interpreted to mean that furosemide eliminated the high-affinity phase of enhancement by allopregnanolone (Fodor *et al.*, 2005) attributed to $\alpha_6\beta\delta$ $GABA_A$ receptors and the remaining low-affinity enhancement associated with α_1 and/or γ_2 subunit-containing $GABA_A$ receptors is less sensitive to inhibition by HBAO. The cerebellar, cortical and

hippocampal inhibitory effects of HBAO around its solubility limit (3 μM) might be attributed to $GABA_A$ receptors without α_6 and/or δ subunits.

$GABA_A$ receptors of the $\alpha_6\beta\delta$ type constitute a major subunit combination of rodent cerebellar $GABA_A$ receptors (Pöltl *et al*., 2003). They are extrasynaptic (Stell *et al*., 2003; Farrant and Nusser, 2005) and they have a functional role in tonic inhibition, tuning motor activity and alcohol-induced motor impairment in cerebellum (Hamann *et al*., 2002; Chadderton *et al*., 2004; Hanchar *et al*., 2005). Moreover, reduced activity of GABA uptake transporters can increase the contribution of tonic inhibition in cerebellar granule cells via $\alpha_6\beta\delta$ $GABA_A$ receptors as a compensatory mechanism observed recently in mice lacking α_1 subunits (Ortinski *et al*., 2006). The two synthesizing enzymes of allopregnanolone and 5α-THDOC are present in different neurons including cerebellar granule cells (Agis-Balboa *et al*., 2006). Physiological (nanomolar) concentrations of these neurosteroids (Concas *et al*., 1998) are compatible with their high affinities to $GABA_A$ receptors containing α_6 and δ subunits (Wohlfarth *et al*., 2002; Bianchi and Macdonald, 2003). Therefore it is reasonable to conclude that these neurosteroids can contribute to the (patho)physiological modulation of cerebellar motor learning and memory storage of sensory information (Chadderton *et al*., 2004; Semyanov *et al*., 2004), the plasticity of $GABA_A$ receptors during stress, oestrous cycle (Maguire *et al*., 2005), pregnancy (Concas *et al*., 1998) and development (Crossley *et al*., 2000). Further, neurosteroids increase the gating efficacy of partial agonists of $\alpha\beta\delta$ $GABA_A$ receptors (Bianchi and Macdonald, 2003), thereby enhancing the contribution of taurine to GABAergic neurotransmission. HBAO might be used as a selective molecular tool to elucidate these processes.

Another important, preferential combination of neurosteroid-sensitive δ subunits are extrasynaptic $\alpha_4\beta\delta$ $GABA_A$ receptors in hippocampus (Adkins *et al*., 2001; Semyanov *et al*., 2004; Farrant and Nusser, 2005; Maguire and Mody, 2007). However, hippocampal [^{3}H]EBOB binding was not displaced by GABA, allopregnanolone (Maksay and Bíró, 2005) and HBAO, each up to 1 μM. This suggests subtype selectivity of HBAO for $\alpha_6\beta\delta$ versus $\alpha_4\beta\delta$ $GABA_A$ receptors. However, the contribution of $\alpha_4\beta\delta$ $GABA_A$ receptors cannot be excluded, since this subtype appears to have a minor population in hippocampus.

Steric structure–activity relationships of neuroactive steroids and $GABA_A$ receptors

Chirality at C3, C5 and C17 strongly affects the interactions of neuroactive steroids with $GABA_A$ receptors. The 3-hydroxylation is essential: ionophore activity is potentiated by 3α-hydroxylated steroids, while it is inhibited by 3β-hydroxylated ones (Lundgren *et al*., 2003) and their anionic esters (Park-Chung *et al*., 1999) noncompetitively. Chirality at C5 determines the A/B ring annellation and the overall shape of the steroid framework. Consequently, it is not surprising that (1) 5α- and 5β-reduced pregnanes have different enantioselectivities (Covey *et al*., 2001); (2) they can be distinctly antagonized by (3α,5α)-17-phenylandrost-16-en-3-ol (Mennerick *et al*., 2004); (3) 5β-THDOC has lower potentiating efficacy than 5α-THDOC (Xue *et al*., 1997); and (4) 5β-, but not 5α-reduced cortisol exhibits antagonist properties (Penland and Morrow, 2004). On the other hand, 5β-THDOC (1 μM) antagonized the nanomolar, but not the micromolar, phase of displacement by 5α-THDOC, while *per se* it did not affect cerebellar [^{3}H]EBOB binding (Maksay and Bíró, 2005). Consequently, high- and low-affinity bindings of THDOC have differential stereoselectivities for chirality at C5.

The 17β-substituents with hydrogen bond accepting groups have also proved to be important for neuroactive steroids (Covey *et al*., 2001). The 17-phenyl group enables (3α,5α)-17-phenylandrost-16-en-3-ol to antagonize 5α-reduced neurosteroids (Mennerick *et al*., 2004). However, micromolar potency and no subunit-selectivity have been reported for this antagonist. The design and synthesis of conformationally constrained, unsaturated 17β-derivatives of allopregnanolone (Souli *et al*., 2005) has led to HBAO. With its chiral 17β-(1-hydroxy-2,3-butadienyl) group, HBAO shows stereoselectivity and nanomolar affinity to a cerebellar subpopulation of $GABA_A$ receptors. Its steric structure is almost identical with those of allopregnanolone and THDOC (Figure 1). The functional differences can be entirely attributed to the conformationally constrained 17β-substituent. This strongly suggests binding sites in common with neurosteroids. It should be noted that allopregnanolone, unlike 5β-reduced pregnanes, has not shown any heterogeneity in displacement of [^{35}S]TBPS and [^{3}H]EBOB bindings (Hawkinson *et al*., 1994; Fodor *et al*., 2005) in various brain regions. Further, the potencies of all neurosteroid antagonists of $GABA_A$ receptors reported previously have been micromolar (Lundgren *et al*., 2003; Mennerick *et al*., 2004). Thus, nanomolar antagonism by HBAO also suggests binding sites common with 5α-reduced neurosteroids potentiating $GABA_A$ receptors in nanomolar concentrations.

Two distinct binding sites of neurosteroids have been recently localized on $GABA_A$ receptors: one in a cavity between transmembrane (TM) regions of the α subunit affecting potentiation and another one in the GABA-binding interface participating in the direct gating of recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors (Hosie *et al*., 2006). Removal of the polar residues by mutations Q241W, N407A and Y410F in TM1 and TM4 of α_1 subunits attenuated only potentiation by THDOC and allopregnanolone supporting hydrogen bonding of the C_{20} ketone group to the polar side chains (Hosie *et al*., 2006). These residues are conserved in α_{1-6} subunits having highly homologous TM regions. Consequently, there are only a few distinct residues in α_6 subunits that can account for the distinctive high-affinity interactions of HBAO. These residues are supposed to form distinct hydrogen bonds with the conformationally constrained hydroxyl group of the 17β-substituent of HBAO instead of the C_{20} ketone of allopregnanolone.

In conclusion, HBAO has rather unprecedented high affinity as an antagonist of neurosteroids potentiating $GABA_A$ receptors. Its nanomolar binding affinity and selectivity might contribute to the localization of the binding sites of potentiating neurosteroids on $\alpha_6\beta\delta$ $GABA_A$ receptors. Further, HBAO might be used as a selective molecular tool to elucidate the neurophysiological processes associated with cerebellar $\alpha_6\beta\delta$ $GABA_A$ receptors.

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Conflict of interest

The authors state no conflict of interest.

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