A Synthetic 18-Norsteroid Distinguishes between Two Neuroactive Steroid Binding Sites on GABA_A Receptors

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ABSTRACT

In the absence of GABA, neuroactive steroids that enhance GABA-mediated currents modulate binding of [35S]-butylbicyclophosphorothionate in a biphasic manner, with enhancement of binding at low concentrations (site NS1) and inhibition at higher concentrations (site NS2). In the current study, compound (3α,5β,17β)-3-hydroxy-18-norandrostane-17-carbonitrile (3α,5β-18-norACN), an 18-norsteroid, is shown to be a full agonist at site NS1 and a weak partial agonist at site NS2 in both rat brain membranes and heterologously expressed GABA_A receptors. 3α,5β-18-norACN also inhibits the action of a full neurosteroid agonist, (3α,5α,17β)-3-hydroxy-17-carbonitrile (3α5αACN), at site NS2. Structure-activity studies demonstrate that absence of the C18 methyl group and the 5β-reduced configuration both contribute to the weak agonist effect at the NS2 site. Electrophysiological studies using heterologously expressed GABA_A receptors show that 3α5β-18-norACN potently and efficaciously potentiates the GABA currents elicited by low concentrations of GABA but that it has low efficacy as a direct activator of GABA_A receptors. 3α5β-18-norACN also inhibits direct activation of GABA_A receptors by 3α5αACN. 3α5β-18-norACN also produces loss of righting reflex in tadpoles and mice, indicating that action at NS1 is sufficient to mediate the sedative effects of neurosteroids. These data provide insight into the pharmacorequired for neurosteroid efficacy at the NS2 site and may prove useful in the development of selective agonists and antagonists for neurosteroid sites on the GABA_A receptor.

Certain endogenous pregnane steroids and their synthetic analogs modulate the function of GABA_A receptors (Lambert et al., 2003). These neuroactive steroids potentiate the actions of GABA at low concentrations and directly open GABA_A receptor channels at higher concentrations (Barker et al., 1987; Cottrell et al., 1987). Several lines of evidence suggest that multiple neuroactive steroid recognition sites contribute to GABA_A receptor modulation. Radioligand binding studies have shown that pregnanolone [(3α,5β)-3-hydroxypregnan-20-one] modulation of [35S]-butylbicyclophosphorothionate (TBPS), [3H]flunitrazepam, and [3H]muscimol binding to GABA_A receptors has two distinct components (Hawkinson et al., 1994b; Hauser et al., 1995). Likewise, in the absence of added GABA, alphaxalone produces biphasic modulation of [35S]TBPS binding: at 10 to 100 nM alphaxalone, [35S]TBPS binding is stimulated; whereas at 1 to 30 µM, it is inhibited (Srinivasan et al., 1999). The two-component modulation of radioligand binding is observed in heterologously expressed receptor preparations as well as in native tissues in which there are GABA_A receptors of various...
subunit compositions. This indicates that two-site behavior is not merely a reflection of heterogeneous subunit expression but rather an indication that GABA_A receptors of defined subunit composition have multiple sites at which neuroactive steroids can modulate function. Recent work using site-directed mutagenesis has identified two putative neurosteroid binding sites, one that mediates potentiation of the effects of GABA and one site that mediates direct activation of the GABA_A receptor (Hosie et al., 2006, 2009). However, the relationship between the two neurosteroid sites observed with radioligand binding and the two sites observed with electrophysiology (coupled with site-directed mutagenesis) has not been rigorously examined.

In this study, we describe the actions of compound 3a,5β,17β-3-hydroxy-18-norandrostane-17-carboxitrite (3a,5β-18-norACN), a neuroactive 18-nor-steroid that preferentially affects one of the two neurosteroid binding sites observed in both [35S]TBPS binding assays and electrophysiological assays. The study also examines the structure-activity relationships underlying site selectivity. Finally, we examined the anesthetic efficacy of compound 3a,5β-18-norACN to determine whether the anesthetic actions of neurosteroids require agonism at both neurosteroid sites.

Materials and Methods

Prepared Materials. The synthesis, spectroscopic and physical properties of 3a,5α-ACN, 3a,5β-ACN, 3a,5α-19-norACN, 3a,5β-19-norACN, 3a,5α-19-nor-P, and 3a,5β-19-nor-P were reported by us previously (Hu et al., 1993; Han et al., 1996). The 18-nor and 18,19-dinosteroids were prepared by similar multistep synthetic procedures. In brief, the 18-methyl group was removed from either a 17-ketosteroid or 19-nor-17-ketosteroid precursor to give the corresponding 18-nor or 18,19-dinosteroids. The seven-step procedure required for removal of the 18-methyl group has been described by us for the preparation of other 18,19-dinor-17-ketosteroids (Han and Covey, 1996). The 17-keto group of the 18-nor or 18,19-dinosteroids was then converted in two steps into the 17-carboxitrite group using a procedure we described previously (Han et al., 1996). Conversion of the 17-carboxitrite group into the acetyl group of 20-ketopregnanes was also described by us previously (Han et al., 1996). The spectroscopic and physical properties of the previously unknown 18-nor and 18, 19-dinosteroids used in this study are given below.

3a,5α-18-norACN. Colorless crystals (from ethyl acetate/hexanes), m.p. 157–159°C; IR 3413, 2238 cm⁻¹; 1H NMR δ 8.05 (m, 1H, CH(OH)), 2.32 to 2.22 (m, 1H, CHCN); 0.75 (s, 3H, CH₃); 13C NMR δ 122.83 (CN), 71.51 (C-2), 50.52, 50.67, 41.99, 41.73, 39.21, 36.03, 35.16, 34.42, 32.63, 30.35, 29.51, 28.30, 27.92, 26.57, 26.27, 24.66, 23.10 (CH₃) Anal. Calcd for C₁₉H₂₇NO: C, 79.07; H, 9.95; N, 5.12. Found: C, 79.03; H, 9.76; N, 5.07.

Vector Construction. cDNA constructs for GABA_A receptor subunits were provided by A. Tobin [University of California, Los Angeles, CA (rat α₁)] and D. Weiss [University of Texas, San Antonio, TX (rat β₂)]. The expression constructs for the rat α₁, β₂, γ₂, and rat α₁γ₁β₂ were described previously (Ueno et al., 1996; Darbandi-Tonakbon et al., 2003). The rat β₂F construct was made by polymerase chain reaction mutagenesis inserting the FLAG between amino acids 4 and 5 using the following oligonucleotides: rat β₂F forward, 5’-gattacaagcttgatgcattctttgc-3’ and rat β₂F reverse, 5’-ctggcacgctgtatatcttcagc-3’. All inserts were sequenced through the entire coding region.

Tissue Culture. Quail fibrillar (QT-6) cells were maintained in culture using standard methods and passaged at subconfluent densities. A stably transfected cell line with rat α₁myc was produced in QT-6 cells by standard methods. In brief, QT-6 cells were transfected with the cDNA using the calcium phosphate precipitation method or using Effectene (QIAGEN, Valencia, CA). Cells resistant to G418 were selected. A population of cells expressing high levels of surface FLAG was selected by immunoselection using the anti-FLAG antibody (M2; Sigma-Aldrich, St. Louis, MO) (Chen et al., 1995).

Membrane Preparation. Rat brains were purchased from Pel-Freeze Biologicals (Rogers, AR) and stored until use at −80°C. Cerebellum and brain stem were trimmed from the frozen brains, and the cerebral hemispheres were used. Membranes for structure-activity relationship experiments (Tables 1 and 2) were prepared using microdialysis procedures. In brief, the 18,19-dinosteroids used in this study are given below.

TABLE 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Hill Slope</th>
<th>IC₅₀</th>
<th>Minimal Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a,5α-ACN</td>
<td>0.96</td>
<td>46 ± 4</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>3a,5β-18-norACN</td>
<td>0.75</td>
<td>59 ± 7</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>3a,5α-19-norACN</td>
<td>0.88</td>
<td>76 ± 4</td>
<td>0 ± 1</td>
</tr>
<tr>
<td>3a,5α-18,19-dinorACN</td>
<td>0.87</td>
<td>133 ± 24</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>3a,5β-ACN</td>
<td>0.90</td>
<td>63 ± 7</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>3a,5β-18-norACN</td>
<td>1.27</td>
<td>49 ± 4</td>
<td>41 ± 1</td>
</tr>
<tr>
<td>3a,5β-19-norACN</td>
<td>0.82</td>
<td>22 ± 3</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>3a,5β-18,19-dinorACN</td>
<td>1.05</td>
<td>73 ± 16</td>
<td>31 ± 3</td>
</tr>
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</table>
homogenized using a Teflon pestle in a motor-driven homogenizer. The homogenate was centrifuged for 10 min at 1000g at 4°C and the pellet was discarded. The supernatant was then centrifuged for 45 min at 100,000g. The resultant pellet was then resuspended in distilled water (12 ml/brain) and stirred for 30 min at 4°C. Membranes were then collected by centrifugation for 45 min at 100,000g at 4°C. The pellet was washed twice with buffer (20 mM potassium phosphate and 50 mM KCl, pH 7.5). The membranes were pelleted after each wash by centrifugation for 45 min at 100,000g at 4°C.

After the final centrifugation, membranes were resuspended in assay buffer (10 mM potassium phosphate and 100 mM KCl, pH 7.5) at approximately 5 mg/ml membrane protein and stored at -80°C.

QT-6 cell membranes were prepared as follows. Cells were grown in monolayer culture to 70 to 80% confluence on 15-cm plates. The plates were washed twice with 5 ml of ice-cold phosphate-buffered saline containing 0.1% protease inhibitor cocktail (Sigma-Aldrich). Five milliliters of TEN (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.5, and 0.1% protease inhibitor cocktail) was added to each plate, and cells were scraped from the plate with a rubber cell scraper. The plates were washed with 5 ml of TEN, and the harvested cells were collected by centrifugation for 10 min at 5000g at 4°C. Cells were resuspended in TEN and homogenized using an Ultra-Turrax high-performance disperser (10-5 s bursts at 4°C; Tekmar-Dohrmann, Mason, OH). Membranes were then centrifuged for 30 min at 30,000g at 4°C, resuspended in TEN (2-3 mg protein/ml), and stored in aliquots at -80°C.

**[35S]TBPS Binding.** [35S]TBPS binding assays were performed using previously described methods (Hawkinson et al., 1994b; Covey et al., 2000), with modification. In brief, aliquots of membrane suspension (0.5 mg/ml final steroid concentration) in assay) were incubated with 1 to 2 nM [35S]TBPS (60–100 Ci/mmol; PerkinElmer Life Sciences, Boston, MA) and 5-μl aliquots of steroid in phosphate buffer, pH 7.5, containing 0.5% Me2SO. Nonspecific binding was added to all screening assays and selected assays with GABA-free agonists and antagonists. Five milliliters of TEN (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 1 mM MgCl2, and 5 mM HEPES at pH 7.4). Capped mRNA encoding rat GABA_A receptor α1, β2, and γ2L subunits was transcribed in vitro using the mMESSAGE mMACHINE kit (Ambion, Austin, TX) from linearized pBluescript vectors containing receptor coding regions. Subunit transcripts were injected in equal parts (20–40 ng of total RNA) 8 to 24 h after transcription. Oocytes were incubated up to 5 days at 18°C in ND96 medium containing 96 mM NaCl, 2 mM KCl, 1 mM MgCl2, and 5 mM HEPES at pH 7.4. Capped mRNA encoding rat GABA_A receptor subunits was originally provided by A. Tobin (University of California, Los Angeles (α1)), P. Malherbe (F. Hoffman-La Roche, Basel, Switzerland (β2)), and C. Fraser (National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD (γ2L)).

GABA currents were measured with an OC275 two-electrode voltage-clamp amplifier (Warner Instruments, Hamden, CT) 2 to 5 days after RNA injection in a bath of unsupplemented ND96 medium. Intracellular recording pipettes had a resistance of ~1 MΩ when filled with 3 M KCl. Compounds were simultaneously coapplied with GABA using a gravity-flow perfusion system. Holding potential was -70 mV, and peak current during 20-s drug applications was used for quantification. Data were acquired and analyzed with pCLAMP software (Molecular Devices, Sunnyvale, CA). Statistical differences were determined using a two-tailed Student’s t test.

**Tadpole and Mouse Anesthetic Assay.** Assays for neuroactive steroid-induced loss-of-righting reflex (LRR) in X. laevis tadpoles and in BALB/c mice were performed as described previously (Covey et al., 2000). In brief, groups of 10 early prelamb-bud stage X. laevis tadpoles (Nasco, Fort Atkinson, WI) were placed in 100 ml of oxygenated Ringer’s buffer containing varying concentrations of 3α5β-18-norACN. After 3 h of equilibration the tadpoles paper was rinsed with 4 ml of ice-cold buffer three times and dissolved in 4 ml of ScintiVerse II (Thermo Fisher Scientific, Waltham, MA). Radioactivity bound to the filters was measured by liquid scintillation spectrometry. Each data point was done in triplicate, and all experiments were performed at least three times. The average specific binding values of each triplicate were used for curve fitting and IC50 values (mean ± S.D. of triplicate determinations), and minimal binding is reported for all compounds.

### TABLE 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Hill Slope</th>
<th>IC50</th>
<th>Minimal Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>3α5βP</td>
<td>0.94</td>
<td>69 ± 8</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>3α5β-18-norP</td>
<td>0.90</td>
<td>157 ± 16</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>3α5β-19-norP</td>
<td>0.99</td>
<td>86 ± 7</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>3αβP</td>
<td>0.65</td>
<td>51 ± 16</td>
<td>7 ± 5</td>
</tr>
<tr>
<td>3αβ-18-norP</td>
<td>0.85</td>
<td>35 ± 11</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>3αβ-19-norP</td>
<td>1.01</td>
<td>12 ± 1</td>
<td>9 ± 1</td>
</tr>
</tbody>
</table>

where B is TPBS bound in the presence of steroid at a given concentration, Bmax is control binding, [C] is steroid concentration, IC50 is the half-maximal inhibition, and n is the Hill coefficient.

The curves describing [35S]TBPS binding performed in the absence of GABA were fit to an equation (eq. 2) in which the term for enhanced binding is multiplied by the term for inhibition of binding:

\[
B = \frac{B_{\text{max}}}{1 + \left(\frac{[C]}{IC_{50}}\right)^n}
\]

where B is steroid bound, Z is the starting maximal binding in the absence of steroids, A is the amplitude of the enhancement, K1 is the half-maximal enhancement concentration, K2 is the half-maximal inhibition concentration, and [C] is steroid concentration. All fits were performed using SigmaPlot version 8 (SPSS Inc., Chicago, IL) and Prism (GraphPad Software Inc., San Diego, CA).
were assessed for the LRR and loss-of-swimming reflex (LSR) behavioral endpoints. LRR was defined as failure of the tadpole to right itself within 5 s after being flipped by a smooth glass rod. LSR was defined as failure to initiate swimming after being flipped by a smooth glass rod. Concentration-response curves were fit to the Hill equation using SigmaPlot version 8.0. For the mouse assay, BALB/C mice were injected intravenously through a tail vein with various doses of $3\alpha\delta$-18-norACN in an 8% ethanol, 16% Cremophor EL (Sigma-Aldrich) solution. LRR was defined as inability of mice to right themselves within 5 s after being placed in a prone position. LRR time was measured from the moment mice displayed LRR until they were able to right themselves.

**Results**

**Structures.** The structures of the neuroactive steroid analogs used in this study are shown in Fig. 1. The structural variables were the $5\alpha$- versus $5\beta$-configuration, and the presence or absence of the C18 and/or C19 methyl groups. Structural variables were examined both in steroids with an acetyl group at carbon 17 (naturally occurring neurosteroids) and with a carbonitrile substitution at carbon 17.

**Neuroactive Steroid Modulation of $[^{35}S]$TBPS Binding in Rat Brain Membranes.** The effects of neurosteroids on $[^{35}S]$TBPS binding were examined in the presence of 5 μM GABA (Fig. 2, A and B) and in membranes depleted of GABA (Fig. 2, A and B). Because complete removal of GABA is difficult, experiments were also done examining the effects of one neurosteroid, $3\alpha\delta$ACN, in the presence of GABAzine, a competitive GABA antagonist (Fig. 2A).

$3\alpha\delta$ACN inhibited $[^{35}S]$TBPS binding, in the presence of 5 μM GABA, with an IC$_{50}$ value of 46 ± 4 nM and a Hill slope of 0.96, consistent with inhibition of a single class of binding sites (Majewska et al., 1986). In the absence of GABA, $3\alpha\delta$ACN showed two-component behavior with enhancement of $[^{35}S]$TBPS binding at low concentration (EC$_{50}$ = 40 ± 35 nM) and inhibition of binding at higher concentra-

![Fig. 1.](image1.png)  Structures of two series of neurosteroid analogs based on either a $5\alpha$-reduced or a $5\beta$-reduced steroid backbone. Structural variables include methyl groups at C18 ($R_2$) and C19 ($R_1$) and acetyl versus carbonitrile groups at C17 ($R_3$).

![Fig. 2.](image2.png)  Effects of neuroactive steroids on specific $[^{35}S]$TBPS binding to rat brain membranes in the presence and absence of exogenous GABA. 3αδACN (A) and 3αδACN (C) modulate $[^{35}S]$TBPS binding in a biphasic manner in the absence of GABA, whereas only the inhibition phase is observed in the presence of 5 μM GABA. In the presence of GABAzine (A), both the stimulatory and inhibitory effects of 3αδACN are observed. B, the same data as in A plotted as femtomoles of specific $[^{35}S]$TBPS binding per milligram of membrane protein. D, compound 3αδ-18-norACN is a preferential NS1 site agonist. In the absence of GABA, 3αδ-18-norACN selectively enhances $[^{35}S]$TBPS binding with minimal inhibition at higher concentrations. 3αδ-18-norACN partially inhibits TBPS binding in the presence of 5 μM GABA. Data shown are the means of triplicate determinations for representative experiments.
tions (IC_{50} = 1.4 ± 0.4 μM) (Fig. 2A). We henceforth refer to the high-affinity (enhancement of TBPS binding) site as NS1 and the low-affinity site (inhibition of TBPS binding) as NS2. It is important to note that the addition of 5 μM GABA not only eliminated enhancement of [35S]TBPS binding but also remarkably reduced baseline [35S]TBPS binding (Fig. 2B). In rat brain membranes, 5 μM GABA reduced TBPS binding (in the absence of steroid) by amounts varying from 50 to 85%. In the presence of GABA,zine, baseline TBPS binding was reduced (presumably by antagonizing small amounts of residual GABA) but both the enhancing effects (EC_{50} = 50 ± 22 nM) and inhibitory effects (IC_{50} = 8 ± 2 μM) of 3α5αACN on [35S]TBPS binding were observed (Fig. 2A). It is noteworthy that the IC_{50} (NS2 effect) value for 3α5αACN was shifted to the right in the presence of GABA.zine.

3α5βACN also inhibited [35S]TBPS binding, in the presence of GABA, with an IC_{50} value of 63 ± 7 nM and a Hill slope of 0.9. In the absence of GABA, 3α5βACN enhanced [35S]TBPS binding at low concentrations (EC_{50} = 29 ± 36 nM) and partially inhibited it at higher concentrations (IC_{50} = 10.7 ± 3.6 μM) (Fig. 2C). The endogenous neurosteroids (acetyl at C17) exhibited behavior that was qualitatively similar to the carbonitrile series; in the absence of GABA, 3α5αP stimulated [35S]TBPS binding, with an EC_{50} value of 54 ± 39 nM, and inhibited it, with an IC_{50} = 3.3 ± 0.7 μM; 3αβP had an EC_{50} = 8 ± 4 nM and an IC_{50} = 38.2 ± 10.7 μM (data not shown in the figure).

Compound 3αβ-18-norACN (previously referred to as B285; Akk et al., 2004), a 5β-reduced steroid lacking the 18-methyl group, modulated [35S]TBPS binding in a distinct pattern (Fig. 2D). In the absence of GABA, 3αβ-18-norACN enhanced [35S]TBPS binding, with an EC_{50} of 67.9 ± 11.1 nM. However, it showed barely discernible inhibition of [35S]TBPS binding even at a concentration of 10 μM. In the presence of 5 μM GABA, 3αβ-18-norACN partially inhibited [35S]TBPS binding (IC_{50} = 49 ± 4 nM), with a Hill slope of 1.27 and a minimal binding of 41 ± 1% of control binding. There is no additional effect of 3αβ-18-norACN between concentrations of 0.3 and 10 μM. These data show that 3αβ-18-norACN preferentially acts at the NS1 site and has low potency and/or efficacy at the NS2 site.

**Interactions of 3αβ-18-norACN and 3α5αACN at the NS1 and NS2 Sites.** To determine whether the minimal effect of 3αβ-18-norACN on NS2 results from poor binding or low efficacy, we examined the interaction of 3αβ-18-norACN and 3α5αACN. In Fig. 3A, the experiments were conducted simultaneously using the same membrane preparation and the same radioligand stock to allow comparison of the absolute amount (femtomoles per milligram of protein) of binding. The effect of 3α5αACN (in the absence of GABA) on [35S]TBPS binding was examined in the presence of 3αβ-18-norACN. 3α5β-18-norACN (3 μM), a concentration that provides maximum 3αβ-18-norACN effect (Fig. 2D), occluded the enhancing action of 3α5αACN at NS1, maximally enhancing [35S]TBPS binding and preventing any further enhancement by 3α5αACN (Fig. 3A). This indicates that 3αβ-18-norACN is a full agonist at the NS1 site. The presence of 3 μM 3αβ-18-norACN also produced a modest change in the IC_{50} value of 3α5αACN at NS2 (1.4 ± 0.4 μM without 3αβ-18-norACN and 5.8 ± 4.9 μM in the presence of 3αβ-18-norACN). To further probe the actions of 3αβ-18-norACN at NS2, the inhibitory effects of 3α5αACN on [35S]TBPS binding were examined in the presence of various concentrations of 3αβ-18-norACN (1, 3, 10, and 30 μM). As shown in Fig. 3B, 1 μM 3αβ-18-norACN enhanced and 10 μM 3α5αACN inhibited [35S]TBPS binding. Increasing concentrations of 3αβ-18-norACN added to 10 μM 3α5αACN significantly increased [35S]TBPS binding (p < 0.05, analysis of variance followed by Tukey’s multiple comparison test of the means), presumably by antagonizing the inhibitory effect of 10 μM 3α5αACN. These data indicate that 3αβ-18-norACN binds to both sites NS1 and NS2 but has low efficacy at NS2.

**Effects of Neuroactive Steroids on [35S]TBPS Binding in Heterologously Expressed GABA_{A} Receptors.** To confirm that the NS1 and NS2 sites both reside on a single pentameric GABA_{A} receptor, steroid modulation of [35S]TBPS binding was examined in cell membranes expressing defined combinations of GABA_{A} receptor subunits. These studies used α_{1}β_{2} heteropentamers. Based on the poor ability of β_{2} subunits to form homopentamers (Bracamontes and Steinbach, 2008), this combination maximizes the likelihood of working with homogeneous populations of GABA_{A} receptors. Membranes from QT-6 cells expressing α_{1}β_{2} GABA_{A} receptor subunits were modulated by 3α5αACN in a manner very similar to that observed in rat brain membranes. In the absence of GABA, 3α5αACN stimulated TBPS binding at low concentrations (EC_{50} = 28 ± 14 nM) and inhibited at higher concentrations (IC_{50} = 537 ± 115 nM). In the presence of GABA (1 μM), 3α5αACN inhibited TBPS binding, with an IC_{50} value of 20 ± 9 nM and a Hill slope of 1 (Fig. 4A). 3αβ-18-norACN appeared to be a selective NS1 agonist in α_{1}β_{2}
IC50 value of 20 partially (65% inhibition) inhibited TBPS binding, with an
inhibition curve. In the C17-carbonitrile series (Table 2), a similar effect of the 18-nor and
C17-acetyl series (Table 2), a similar effect of the 18-nor and
5-reduced configurations was observed. It is noteworthy
that pregnanolone (3\beta) almost completely inhibited
[T35S]TBPS binding (IC50 = 20 ± 9 nM). B, in the absence of GABA, 3\beta-18-norACN selectively
enhances [35S]TBPS binding to \(\alpha_1\beta_2\) receptors (EC50 = 50 ± 16 nM); in the presence of 1 μM GABA, enhancement is eliminated and
3\beta-18-norACN only inhibits TBPS binding (IC50 = 20 ± 9 nM). C, GABA modulates
[35S]TBPS binding to \(\alpha_{1\text{myc}}\beta_{2\text{FLAG}}\) receptors in a biphasic manner (EC50 = 119 ± 1 nM; IC50 = 120 ± 1 nM).

**Structural Requirements for Steroids Providing Low Efficacy at the NS2 Site.** To determine which structural properties of 3\beta-18-norACN cause it to have low efficacy at the NS2 site, [35S]TBPS binding (in the presence of 5 μM GABA) was performed with all of the compounds shown in Fig. 1. The data were fit to a one-component inhibition curve. In the C17-carbonitrile series (Table 1), all steroids lacking the 18-methyl group failed to completely inhibit [35S]TBPS binding (minimal binding >10%); in contrast all steroid containing the 18-methyl group completely inhibited TBPS binding. This effect of the 18-nor compounds was much more pronounced in the 5\beta-reduced steroids than in the 5α-reduced steroids. Absence of the 19-methyl group affected neither minimal binding nor the Hill slope. In the C17-acetyl series (Table 2), a similar effect of the 18-nor and 5\beta-reduced configurations was observed. It is noteworthy that pregnanolone (3\betaP) almost completely inhibited [35S]TBPS binding but did so with a Hill slope of 0.65, suggesting the possibility of two-component inhibition. These data indicate that the absence of the 18-methyl group and the 5\beta-reduced configuration both contribute to lack of neurosteroid efficacy at the NS2 site.

**Electrophysiological Effects of 3\beta-18-norACN.** The ability of 3\beta-18-norACN, 3\alpha5\alphaACN, and 3\beta5\betaACN to potentiate GABA-elicited (2 μM) currents and to directly activate GABA receptors was examined in *X. laevis* oocytes expressing \(\alpha_1\beta_2\) GABA \(\alpha\) receptor subunits. Figure 5A shows superimposed traces of representative currents elicited by 2 μM GABA alone (the lowest amplitude trace) and 2 μM GABA plus 0.1, 1, or 10 μM 3\beta-18-norACN, 3\beta5\betaACN, or 3\alpha5\alphaACN. The concentration-response curves (Fig. 5B) demonstrate that 3\alpha5\alphaACN (\(E_{\text{max}} = 14 ± 21\)) has modestly higher efficacy than 3\beta-18-norACN (\(E_{\text{max}} = 10 ± 0.3\) or...
3α5βACN ($E_{max} = 7.8 \pm 1.6$) in potentiating GABA-elicited currents. However, there is no statistical difference among them. Two-way analysis of variance indicated that 0.3, 1, and 3 μM 3α5αACN had higher potentiation than 3α5β-18-norACN and 3α5βACN. However, there was no difference among these three neurosteroids at 10 μM. 3α5β-18-norACN, 3α5βACN, and 3α5αACN have similar potency for potentiating GABA responses, with $EC_{50}$ values of 0.6 ± 0.1, 1 ± 0.4, and 0.2 ± 0.4 μM, respectively. These results indicate that there is not a major difference in potency or efficacy among 3α5αACN, 3α5β-18-norACN, and 3α5βACN in potentiating GABA-elicited currents.

The ability of 3α5β-18-norACN, 3α5αACN, and 3α5βACN to directly activate GABA$_A$ receptors in the absence of GABA was also examined. To decrease differences among oocytes, the direct gating currents were normalized to currents elicited by 2 μM GABA in the same cell. Figure 6A shows representative traces of steroid-elicited currents in comparison with 2 μM GABA. As shown in Fig. 6B, 3α5β-18-norACN elicited very small GABA currents compared with 3α5αACN; 30 μM 3α5β-18-norACN gated a current 1.7 ± 0.2% as large as that elicited by 2 μM GABA. Based on this low-efficacy concentration-response curve (Fig. 6B, bottom), the observed $EC_{50}$ value of 3α5β-18-norACN for direct gating was 1.6 ± 0.4 μM. 3α5βACN at 30 μM elicited currents equal to 5.0 ± 0.2% of the 2 μM GABA currents, with an $EC_{50}$ value of 3.3 ± 0.2 μM. 3α5αACN showed much higher gating efficacy; 30 μM 3α5αACN elicited currents were as large as 34 ± 2.4% of 2 μM GABA. The $EC_{50}$ value of 3α5αACN could not be accurately determined because maximal effect was not achieved at concentrations that maintained solubility.

The low efficacy of 3α5β-18-norACN, coupled with its relatively high apparent potency as a direct activator of GABA$_A$ currents suggested that it might antagonize the actions of more efficacious neurosteroids at the site mediating direct activation. To test this idea, we examined the ability of 10 μM 3α5αACN to directly activate currents in the presence and absence of 30 μM 3α5β-18-norACN. The currents elicited by 10 μM 3α5αACN and 30 μM 3α5β-18-norACN were 0.40 ± 0.05 and 0.03 ± 0.01 μA, respectively (Fig. 6C). 3α5β-18-norACN at 30 μM dramatically decreased the current elicited by 10 μM 3α5αACN. In the presence of 3α5β-18-norACN, the current elicited by 10 μM 3α5αACN was 0.10 ± 0.02 μA (Fig. 6D, ***p < 0.001 versus 3α5αACN alone). These results are consistent with competition between 3α5β-18-norACN and 3α5αACN at the direct activation site.

Anesthetic Effects of 3α5β-18-norACN in Tadpoles and Mice. To test the anesthetic effects of a relatively selective NS1 agonist, two behavioral endpoints, LRR and LSR were examined in *X. laevis* prelibium-bud stage tadpoles exposed to varying concentrations of 3α5β-18-norACN. 3α5β-18-norACN caused LRR, with an $EC_{50}$ value of 164 ± 40 nM (Fig. 7A). Our previous work showed that the $EC_{50}$ values for LRR by 3α5αACN, 3α5αP, 3α5βACN, and 3α5βP in tadpoles were 70 ± 10, 390 ± 40, 80 ± 13, and 61 ± 4 μM, respectively (Wittmer et al., 1996; Covey et al., 2000). They are not statistically significantly different compared with 3α5β-18-norACN. 3α5β-18-norACN at 1.0 μM caused no LSR. However, 3 and 10 μM 3α5β-18-norACN caused LSR in all tadpoles. The ability of 3α5β-18-norACN to anesthetize mice was also examined. 3α5β-18-norACN produced loss of righting reflex at a threshold dose of ~9 mg/kg i.v. This is similar to the threshold dose of 4 mg/kg for 3α5αACN to produce loss of
righting reflex (Wittmer et al., 1996). The duration of loss of righting reflex was dose-dependent (Fig. 7B).

Discussion

This study demonstrates that compound 3α5β-18-norACN (previously named B285; Akk et al., 2004), a 5β-reduced steroid lacking the 18-methyl group, binds to steroid sites NS1 and NS2 on GABA<sub>A</sub> receptors, acting as an agonist at the NS1 site and as a weak partial agonist at the NS2 site. Both the 5β-reduced configuration and the absence of the 18-methyl group contribute to the low efficacy of 3α5β-18-norACN at the NS2 site. 3α5β-18-norACN also selectively potentiates GABA-elicited currents but produces minimal direct activation of GABA<sub>A</sub> receptor currents; it appears to be a weak partial agonist at the site mediating direct activation of GABA<sub>A</sub> receptors, as it reduces the direct activation of GABA<sub>A</sub> currents elicited by 3α5αACN.

Action of Neuroactive Steroids on TBPS Binding. 35S]TBPS (a cage convulsant that binds at the picrotoxin site on GABA<sub>A</sub> receptors) binding is a useful reporter for the actions of allosteric modulators of GABA<sub>A</sub> receptors. In well washed brain membranes and in recombinant GABA<sub>A</sub> receptors, 3α5αACN enhances [35S]TBPS binding at low concentrations and inhibits it at higher concentrations (Figs. 2 and 4), consistent with previous observations of similar actions of allopregnanolone ([α,5α]-3-hydroxypregnan-20-one), pregnanalone, and alphaxalone (Davies et al., 1997; Srinivasan et al., 1999). We have designated the neuroactive steroid binding site mediating enhancement as NS1 and the inhibitory site as NS2 to distinguish them from sites A and B described using single channel recording (Akk et al., 2004, 2009; Bracamontes and Steinbach, 2009). Sites NS1 and NS2 do not represent steroid binding sites on distinct GABA<sub>A</sub> receptors differing in subunit composition, because both sites are observed in recombinant α1β2 GABA<sub>A</sub> receptors (Davies et al., 1997) (Fig. 4A), a combination in which neither subunit expresses well as a homomeric receptor (Bracamontes and Steinbach, 2008).

The biphasic actions of neuroactive steroids on [35S]TBPS binding can be explained using a conceptual model (Fig. 8) in which there are two GABA binding sites and two classes of neuroactive steroid binding sites (NS1 and NS2). It is important to note that the stoichiometry of neurosteroid binding and TBPS binding is not addressed in this model. The stoichiometry of TBPS (picrotoxin) binding to GABA<sub>A</sub> receptors is not precisely known. Although there may be multiple NS1 and/or NS2 sites on a pentameric GABA<sub>A</sub> receptor, we have not included NS1 or NS2 stoichiometry in our model, thus making the implicit assumption that occupancy of a single NS1 or NS2 site is sufficient to produce the full effect. Finally, our model assumes that as GABA concentration is increased, GABA sequentially occupies its two binding sites. This implies that at low GABA concentration monoligated receptors will predominate, whereas diliganded receptors will be the principal species at high GABA concentrations. The above-mentioned assumptions about TBPS and neurosteroid (NS1 and NS2) stoichiometry and GABA site ligation are limitations of our model, which need to be validated before this model can be considered more than a conceptual framework that describes our data.

In the absence of bound GABA or neurosteroid, the receptor (R) can bind [35S]TBPS (Fig. 8). When the receptor is monoligated with GABA (RG) or site NS1 is occupied (RS<sub>1</sub>), its affinity for TBPS is increased, resulting in increased [35S]TBPS binding. In receptors that are monoligated with both GABA and S1 (RS<sub>1S</sub>), TBPS binding is partially inhibited. This partial inhibition is most apparent when 3α5β-18-norACN occupies site NS1, because it has minimal efficacy at site NS2. When receptors are diliganded at either both GABA sites (RGG) or both steroid sites (RS<sub>1S</sub>S<sub>2</sub>), TBPS binding is completely inhibited.

Fig. 7. Anesthetic effects of 3α5β-18-norACN in X. laevis tadpoles and BALB/c mice. A, compound 3α5β-18-norACN caused LRR and LSR in tadpoles. Points on the tadpole concentration-response curves represent 10 to 20 animals, scored quantally. The EC<sub>50</sub> value for LRR was 164 ± 40 nM (S.E.). 3α5β-18-norACN at 0.3 μM has no effect on LSR, whereas 1 and 3 μM 3α5β-18-norACN produce LSR in all the tadpoles. B, intravenous injection of 3α5β-18-norACN produced dose-dependent LRR (sleep times) in mice. Points ± S.E. on the mouse dose-response curve represent the average sleep time for three or four animals and were fit to a straight line.

Fig. 8. Model of neurosteroid and GABA modulation of [35S]TBPS binding. In the absence of bound GABA or neurosteroid, the receptor (R) can bind [35S]TBPS. When the receptor is monoligated with GABA (RG) or site NS1 is occupied (RS<sub>1</sub>), its affinity for TBPS is increased, resulting in increased [35S]TBPS binding. In receptors that are monoligated with both GABA and S1 (RS<sub>1S</sub>), TBPS binding is partially inhibited. This partial inhibition is most apparent when 3α5β-18-norACN occupies site NS1, because it has minimal efficacy at site NS2. When receptors are diliganded at either both GABA sites (RGG) or both steroid sites (RS<sub>1S</sub>S<sub>2</sub>), TBPS binding is completely inhibited.
plied (RS2,G). [35S]TBPS binding is partially inhibited. This partial inhibition is most apparent when 3αβ-18-norACN occupies NS1, because it has minimal efficacy at NS2 (Figs. 2D and 4B). Finally, when the receptor is either diligated with GABA (RGG) or both NS1 and NS2 are occupied (RS2,S), the receptors are unable to bind [35S]TBPS.

**Action of 3αβ-18-norACN on TBPS Binding.** 3αβ-18-norACN behaves as a selective NS1 site agonist. In the absence of GABA (RS2,G in Fig. 8), it enhances TBPS binding at low concentrations and minimally inhibits TBPS binding at higher concentrations (Fig. 2D). 3αβ-18-norACN also occludes the NS1 actions of 3α5aACN, indicating that these two ligands compete for binding at site NS1 and have similar efficacy (Fig. 3A). In contrast, although 3αβ-18-norACN alone produces no NS2 site effect, increasing concentrations of 3αβ-18-norACN antagonize the actions of 3α5aACN as an NS2 site agonist (Fig. 3D); this suggests that 3αβ-18-norACN occupies the NS2 site but has minimal efficacy.

In the presence of 5 μM GABA (Fig. 2), both the NS1 and NS2 sites contribute to complete inhibition of TBPS binding. 3αβ-18-norACN only partially inhibits TBPS binding indicating that it lacks the NS2 site effect (Figs. 2D and 4B; RS2,G in Fig. 8). An 18-methyl group and a 5β-reduced configuration were identified as important contributors to agonist efficacy at the NS2 site. Neurosteroids lacking the 18-methyl and/or having a 5β-reduced configuration can bind to the NS2 site but have minimal agonist activity. Collectively, these data confirm that the NS1 and NS2 sites are nonidentical and indicate the feasibility of developing selective agonists and antagonists for these distinct steroid binding sites. Several other steroid analogs with the 5β-reduced configuration, including the 3,20-pregnandiones and (3α,5β)-3,21-dihydropregn-20-one, have also been shown to partially inhibit [35S]TBPS binding in the presence of GABA and have been classified as partial agonists (Belelli and Ghe, 1989; Morrow et al., 1990; Hawkinson et al., 1996; Xue et al., 1997). These compounds may also be selective ligands for either the NS1 or NS2 sites.

**Electrophysiological Action of 3αβ-18-norACN.** At a macroscopic level, 3αβ-18-norACN potentiates GABA-elicited currents with potency and efficacy similar to that of 3α5aACN (Fig. 5). 3αβ-18-norACN shows minimal efficacy as a direct activator of GABA<sub>α</sub> receptors (α<sub>1β<sub>1γ<sub>2</sub></sub>) expressed in X. laevis oocytes (Fig. 6). Furthermore, 3αβ-18-norACN antagonizes the direct activation elicited by 3α5aACN, suggesting that it is a weak partial agonist at the site mediating direct activation of GABA<sub>α</sub> receptors.

**Relationship between Neurosteroid Binding Sites Identified in Radioligand Binding and Electrophysiological Assays.** Two distinct concentration-dependent effects of neurosteroids are observed in [35S]TBPS binding assays. We have interpreted these data as indicative of two distinct (NS1 and NS2) neurosteroid binding sites on the GABA<sub>α</sub> receptor. Neurosteroids also produce two distinct concentration-dependent effects in electrophysiological assays: potentiation of GABA-responses at low neurosteroid concentrations and direct activation of the GABA<sub>α</sub> receptor at high concentrations. Site-directed mutagenesis studies indicate that these electrophysiological effects are mediated by two neurosteroid binding sites, one site that mediates potentiation and one site that mediates direct activation (Hosie et al., 2006). Although the relationship between the sites observed in radioligand binding studies and electrophysiological studies is not defined, it is simple and attractive to consider that the two assays are describing the same sites with NS1 corresponding to the potentiation site and NS2 corresponding to the direct activation site. 3αβ-18-norACN provides some evidence in support of this hypothesis: the concentrations of 3αβ-18-norACN that activate NS1 correspond closely with those that produce potentiation of GABA-elicited currents. 3αβ-18-norACN is also a poor agonist at the NS2 site in the TBPS binding assay and a weak direct activator of GABA<sub>α</sub> currents. Finally, 3αβ-18-norACN prevents the actions of 3α5α-ACN as an NS2 agonist in TBPS binding and as a direct activator of GABA<sub>α</sub> currents. Although these data support the hypothesis that the NS1 and NS2 sites are synonymous with the potentiating and direct activating sites, more extensive studies are required to confirm these assignments. Specifically, parallel examination of a larger set of neurosteroids in [35S]TBPS binding assays and whole cell electrophysiological assays in both wild-type GABA<sub>α</sub> receptors and receptors in which the potentiation and activation sites have been mutated would provide a more thorough test of this hypothesis.

Akk and colleagues have provided evidence for multiple neurosteroid binding sites (A, B1, and B2) in single channel electrophysiological studies using recombinant α<sub>1β<sub>1γ<sub>2</sub></sub>-subunit GABA<sub>α</sub> receptors (Akk et al., 2004; Li et al., 2007). Their studies were performed with 50 μM GABA, a concentration at which most receptors are diligated with GABA and thus not observable with [35S]TBPS binding. In our model, the neurosteroid effects they observe would correspond to states in which the receptor is diligated with GABA and sites NS1, NS2, or both are occupied. None of these sites could be observed with [35S]TBPS binding. There is thus no basis for correlating the multiple neurosteroid binding sites characterized by single channel electrophysiology with sites NS1 and NS2 demonstrated in this study.

**Anesthetic Action of 3αβ-18-norACN.** Compound 3αβ-18-norACN also provides a tool for understanding the biological actions of neurosteroids at the NS1 and NS2 sites. 3αβ-18-norACN produces loss-of-righting reflex in X. laevis tadpoles and in mice (Fig. 7). The loss-of-righting reflex and loss-of-swimming reflex effects of 3αβ-18-norACN have a similar concentration dependence to those of 3α5α-ACN and 3αβ-ACN (Wittmer et al., 1996; Covey et al., 2000). Because 3α5aACN, 3αβ-ACN, and 3αβ-18-norACN all have similar efficacy at NS1 and as potentiators of GABA-elicited currents and 3αβ-18-norACN has minimal efficacy at NS2 or as a direct activator of GABA<sub>α</sub> receptors, these data indicate that efficacy at NS1 and potentiation of GABA-elicited currents is sufficient for a neurosteroid to produce loss-of-righting reflex.

In summary, the behavior of 3αβ-18-norACN in radioligand binding and electrophysiological assays increases the evidence that there are two classes of neurosteroid binding sites on GABA<sub>α</sub> receptors that can be distinguished by selected neurosteroid ligands. The initial description of the structure-activity requirements for efficacy at these sites should provide impetus for the development of selective agonist and antagonists for the two neurosteroid sites. The development of such selective neurosteroid ligands will be a
A Site-Selective Neurosteroid Ligand

A site-selective neurosteroid ligand can be a vital tool for elucidating the biological actions of endogenous neurosteroids and may provide useful clinical agents.

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