A Synthetic 18-Norsteroid distinguishes between two neuroactive steroid binding sites on GABA_A receptors

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**Abbreviations:** 3α5αACN, (3α,5α,17β)-3-Hydroxyandrostane-17-carbonitrile; 3α5βACN, (3α,5β,17β)-3-Hydroxyandrostane-17-carbonitrile; 3α5β-18-norACN, (3α,5β,17β)-3-Hydroxy-18-norandrostane-17-carbonitrile; 3α5α-18-norACN, (3α,5α,17β)-3-Hydroxy-18-norandrostane-17-carbonitrile; 3α5β-19-norACN, (3α,5β,17β)-3-Hydroxyestrane-17-carbonitrile; 3α5α-19-norACN, (3α,5α,17β)-3-Hydroxyestrane-17-carbonitrile; 3α5β-19,19-dinorACN, (3α,5β,17β)-3-Hydroxyestrane-17-carbonitrile; 3α5β-18,19-dinorACN, (3α,5β,17β)-3-Hydroxyestrane-17-carbonitrile; 3α5α-18,19-dinorACN, (3α,5α,17β)-3-Hydroxygonane-17-carbonitrile; 3α5αP, (3α,5α)-3-Hydroxypregnan-20-one; 3α5βP, (3α,5β)-3-hydroxypregnan-20-one; 3α5β-18-norP, (3α,5β,17β)-3-Hydroxy-18-norpregnan-20-one; 3α5α-18-norP, (3α,5α,17β)-3-Hydroxy-18-norpregnan-20-one; 3α5β-19-norP, (3α,5β,17β)-3-Hydroxy-19-norpregnan-20-one; 3α5α-19-norP, (3α,5α,17β)-3-Hydroxy-19-norpregnan-20-one; GABA, γ-amino butyric acid; GABA<sub>A</sub> receptor, GABA receptor type A; TBPS, t-butylbicyclopentaphosphorothionate; SEM, standard error of mean
ABSTRACT

In the absence of GABA, neuroactive steroids that enhance GABA-mediated currents modulate binding of [35S]TBPS 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-18-norACN (3α,5β,17β)-3-hydroxy-18-norandrostane-17-carbonitrile, 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<sub>A</sub> receptors. 3α5β-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<sub>A</sub> receptors show that 3α5β-18-norACN potently and efficaciously potentiates the GABA currents elicited by low concentrations of GABA, but has low efficacy as a direct activator of GABA<sub>A</sub> receptors. 3α5β-18-norACN also inhibits direct activation of GABA<sub>A</sub> 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 pharmacophore required 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<sub>A</sub> receptor.
INTRODUCTION

Certain endogenous pregnane steroids and their synthetic analogues 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]t-butylbicyclophosphorothionate (TBPS), [3H]flunitrazepam, and [3H]muscimol binding to GABA$_A$ receptors has two distinct components (Hauser et al., 1995; Hawkinson et al., 1994b). Similarly, in the absence of added GABA, alphaxalone produces biphasic modulation of [35S]TBPS binding: at 10-100 nM alphaxalone, [35S]TBPS binding is stimulated; whereas at 1-30 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 composition. 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 that mediates direct activation of the GABA$_A$ receptor (Hosie et al., 2009; Hosie et al., 2006). 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 3α,5β-18-norACN ((3α,5β,17β)-3-hydroxy-18-norandrostane-17-carbonitrile), a neuroactive 18-norsteroid 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 $3\alpha 5\beta$-18-norACN to determine if the anesthetic actions of neurosteroids require agonism at both neurosteroid sites.

METHODS

Prepared materials: The synthesis, spectroscopic and physical properties of $3\alpha 5\alpha$ACN, $3\alpha 5\beta$ACN, $3\alpha 5\alpha$-19-norACN, $3\alpha 5\beta$-19-norACN, $3\alpha 5\alpha$-19-norP and $3\alpha 5\beta$-19-norP were reported by us previously (Han et al., 1996; Hu et al., 1993). The 18-nor and 18,19-dinorsteroids were prepared by similar multi-step synthetic procedures. Briefly, 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-dinorsteroids. 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-dinorsteroids was then converted in two steps into the 17-carbonitrile group using a procedure we described previously (Han et al., 1996). Conversion of the 17-carbonitrile group into the acetyl group of 20-ketopreganes was also described by us previously (Han et al., 1996). The spectroscopic and physical properties of the previously unknown 18-nor and 18, 19-dinorsteroids used in this study are given below.

$3\alpha 5\alpha$-18-norACN: colorless crystals (from ethyl acetate/hexanes), mp 157–159 °C; IR 3413, 2238 cm$^{-1}$; $^1$H NMR $\delta$ 4.05 (m, 1H, CHO), 2.31–2.21 (m, 1H, CHCN); 0.75 (s, 3H, CH$_3$); $^{13}$C NMR $\delta$ 122.91 (CN), 66.33 (C-3), 52.89, 52.19, 50.64, 41.75, 38.76, 36.05, 35.68, 32.67, 32.17, 31.94, 29.46, 28.91, 28.06, 27.99, 24.64, 11.05 (CH$_3$). Anal. Calcd for C$_{19}$H$_{29}$NO: C, 79.39; H, 10.17, N, 4.87. Found: C, 79.12; H, 10.34, N, 4.68.

$3\alpha 5\beta$-18-norACN: colorless crystals (from ethyl acetate/hexanes), mp 179–81 °C; IR 3401, 2237 cm$^{-1}$; $^1$H NMR $\delta$ 3.64 (m, 1H, CHO), 2.32-2.22 (m, 1H, CHCN), 0.89 (s, 3H, CH$_3$); $^{13}$C NMR $\delta$ 122.83 (CN), 71.51 (C-3), 52.05, 50.67, 41.99, 41.73, 39.21, 36.03, 35.16, 34.42, 32.63, 30.35, 29.51, 28.03, 27.92,

**3α5α-18-norP:** colorless crystals (from ethyl acetate/hexanes), mp 127–128 °C; IR 3295, 1708 cm⁻¹; ¹H NMR δ 4.04 (m, 1H, CHO), 2.54–2.45 (m, 1H, CHCOCH₃), 2.14 (s, 3H, CH₃CO), 0.72 (s, 3H, CH₃); ¹³C NMR δ 212.08 (C=O), 66.39 (C-3), 57.36, 53.08, 48.77, 41.87, 38.83, 36.04, 35.76, 32.19, 32.13, 30.41, 29.65, 28.91, 28.53, 28.22, 26.87, 24.93 11.05 (CH₃). Anal. Calcd for C₂₀H₃₂O₂: C, 78.90; H, 10.59. Found: C, 79.00; H, 10.36.

**3α5β-18-norP:** colorless crystals (from ethyl acetate/hexanes), mp 146–148 °C; IR 3396, 1705 cm⁻¹; ¹H NMR δ 3.65 (m, 1H, CHO), 2.55–2.45 (m, 1H, CHCOCH₃), 2.14 (s, 3H, CH₃CO), 0.88 (s, 3H, CH₃); ¹³C NMR δ 212.13 (C=O), 71.80 (C-3), 57.42, 53.01, 48.82, 42.20, 41.94, 39.41, 36.22, 35.25, 34.51, 30.56, 30.49, 29.58, 28.54, 26.93, 26.77, 26.49, 25.02, 23.22. Anal. Calcd for C₂₀H₃₂O₂: C, 78.90; H, 10.59. Found: C, 79.12; H, 10.63.

**3α5α-18,19-dinorACN:** colorless crystals (from ethyl acetate), mp 174–175 °C; IR 3317, 2238 cm⁻¹; ¹H NMR δ 4.08 (m, 1H, CHO), 2.32–2.23 (m, 1H, CHCN); ¹³C NMR δ 122.89 (CN), 66.10 (C-3), 51.10, 50.72, 47.48, 46.74, 46.56, 40.34, 35.55, 33.20, 32.75, 32.63, 30.87, 29.20, 29.06, 27.95, 27.74, 23.57. Anal. Calcd for C₁₈H₂₇NO: C, 79.07; H, 9.95; N, 5.12. Found: 78.87; H, 9.89; N, 5.09.

**3α5β-18,19-dinorACN:** colorless crystals (from ethyl acetate/hexanes), mp 158–160 °C; IR 3299, 2236 cm⁻¹; ¹H NMR δ 3.69–3.59 (m, 1H, CHO), 2.33–2.23 (m, 1H, CHCN); ¹³C NMR δ 122.80 (CN), 71.46 (C-3), 51.01, 50.80, 48.01, 39.74, 37.11, 36.10, 35.33, 32.78, 31.18, 29.59, 29.38, 29.24, 28.09, 27.93, 26.23, 25.93. Anal. Calcd for C₁₈H₂₇NO: C, 79.07; H, 9.95; N, 5.12. Found: C, 79.03; H, 9.76; N, 5.07.
Vector construction: cDNA constructs for GABAA receptor subunits were provided by A. Tobin, University of California Los Angeles (rat α1) and D. Weiss, University of Texas, San Antonio, TX (rat β2). The expression construct for the rat α1F pcDNA3, rat β2 pcDNA3 and rat α1myc were previously described (Darbandi-Tonkabon et al., 2003; Ueno et al., 1996). The rat β2F construct was made by PCR mutagenesis inserting the FLAG between amino acids 4 and 5 using the oligonucleotides: rat β2F forward: 5’ gattacaaggacgatgacgacaaggaccctagtaatatgtcctgg 3’; and rat β2F reverse: 5’ cttgacgacatcgtctgtatcattgacactctgagcacagc 3’. All inserts were sequenced through the entire coding region.

Tissue culture: Quail fibroblast (QT-6) cells were maintained in culture using standard methods and passaged at subconfluent densities. A stably transfected cell line with rat α1myc rat β2Flag 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) (Chen et al., 1995).

Membrane preparation: Rat brains were purchased from Pel-Freez (Rogers, AK) and stored until use at -80°C. Cerebella 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 minor modifications of the method of Hawkinson (Hawkinson et al., 1994a), as previously described (Covey et al., 2000). For all other studies GABA-depleted membranes were prepared using minor modifications of the method described by Srinivasan et. al (Srinivasan et al., 1999). Briefly, brains were immersed in ice cold 0.32 M sucrose (10 ml/gm) and homogenized using a Teflon pestle in a motor-driven homogenizer. The homogenate was centrifuged for 10 min at 1,000g 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 minutes 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 K-Phosphate, 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 K-Phosphate, 100 mM KCl, pH 7.5) at approximately 5 mg membrane protein/ml and stored at -80°C.

QT-6 cell membranes were prepared as follows: Cells were grown in monolayer culture to 70-80% confluency on 150 cm plates. The plates were washed twice with 5 ml ice-cold phosphate-buffered saline containing 0.1% protease inhibitor cocktail (Sigma Chemical, St. Louis, MO). 5 ml of TEN (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.5, 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 a Tekmar Ultra-Turrax (ten 5-sec bursts at 4°C). Membranes were then collected by centrifugation 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 (Covey et al., 2000; Hawkinson et al., 1994b) with modification. Briefly, aliquots of membrane suspension (0.5 mg/ml final protein concentration in assay) were incubated with 1-2 nM [^35S]TBPS (60-100Ci/mmol, Perkin Elmer Life Science, Boston, MA) and 5 μl- aliquots of steroid in Me₂SO solution (final steroid concentrations ranged from 1 nM to 10 μM), in a total volume of 1 ml of assay buffer. For rat brain membranes the assay buffer was 100 mM KCl, 10 mM K-Phosphate buffer, pH 7.5; for QT-6 cell membranes assay buffer was 20 mM Tris-HCl, 1 M NaCl, pH 7.5; for the structure-activity screens shown in tables, the assay buffer was 50 mM K-phosphate buffer, pH7.4, 200 mM NaCl. GABA (5 μM)
was added to all screening assays and selected assays with GABA-free membranes to analyze its effect on \[^{35}\text{S}]\text{TBPS}\) binding. For experiments shown in Figure 4, 1 \(\mu\text{M}\) GABA was used because it inhibited \(^{35}\text{S}\)TBPS binding by \(\approx 50\%\), whereas 5 \(\mu\text{M}\) GABA completely inhibited specific TBPS binding in QT-6 cells expressing recombinant \(\alpha_1\beta_2\) subunits of the GABA\(_A\) receptors. Control binding was defined as binding observed in the presence of 0.5\% Me\(_2\)SO and the absence of steroid; all assays contained 0.5\% Me\(_2\)SO. Nonspecific binding was defined as binding observed in the presence of 200 \(\mu\text{M}\) picrotoxin and ranged from 12.4 to 32.6\% of total binding. Assay tubes were incubated for 2 hours at room temperature. A Brandel (Gaithersburg, MD) cell harvester was used for filtration of the assay tubes through Whatman glass fiber (GF/C) filter paper. Filter paper was rinsed with 4 ml of ice-cold buffer three times and dissolved in 4 ml ScintiVerse II (Fisher Scientific, Pittsburgh, PA). 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 \(\text{EC}_{50}\) or \(\text{IC}_{50}\) is presented as the parameters of the curve fitting to the pooled data from the repeated experiments ± SEM.

The data from \(^{35}\text{S}\)TBPS binding performed in the presence of GABA were fit to the Hill equation (equation 1).

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

where \(B\) is TBPS bound in the presence of steroid at a given concentration, \(B_{\text{max}}\) is control binding, \([C]\) is steroid concentration, \(\text{IC}_{50}\) is the half-maximal inhibition, and \(n\) is the Hill coefficient.

The curves describing \(^{35}\text{S}\)TBPS binding performed in the absence of GABA were fit to an equation (equation 2) in which the term for enhanced binding is multiplied by the term for inhibition of binding:
where $B$ is steroid bound, $Z$ is the starting maximum binding in the absence of steroids; $A$ is the amplitude of the enhancement, $K_1$ is the half-maximal enhancement concentration, $K_2$ is the half-maximal inhibition concentration, and $[C]$ is steroid concentration.

All fits were performed using Sigma Plot version 8 (SPSS; Chicago IL) and Prism GraphPad (San Diego, CA).

**Xenopus Oocyte electrophysiological methods.** Stage V-VI oocytes were harvested from sexually mature female *X. laevis* (Xenopus One, Northland, MI) under 0.1% tricaine (3-aminobenzoic acid ethyl ester) anesthesia. Oocytes were defolliculated by shaking for 20 min at 37°C in collagenase (2 mg/ml) dissolved in calcium-free solution containing (in mM): 96 NaCl, 2 KCl, 1 MgCl$_2$, and 5 HEPES at pH 7.4. Capped mRNA encoding rat GABA$_A$ receptor $\alpha_1$, $\beta_2$ and $\gamma_{2L}$ subunits was transcribed in vitro using the mMESSAGE mMMachine Kit (Ambion, Austin, TX) from linearized pBluescript vectors containing receptor coding regions. Subunit transcripts were injected in equal parts (20-40 ng total RNA) 8-24 h following defolliculation. Oocytes were incubated up to 5 days at 18°C in ND96 medium containing (in mM) 96 NaCl, 1 KCl, 1 MgCl$_2$, 2 CaCl$_2$, and 5 HEPES at pH 7.4, supplemented with pyruvate (5 mM), penicillin (100 U/ml), streptomycin (100 $\mu$g/ml), and gentamycin (50 $\mu$g/ml). The cDNAs for the rat GABA$_A$-receptor subunits were originally provided by A. Tobin, University of California, Los Angeles ($\alpha_1$); P. Malherbe, Hoffman-La Roche, Switzerland ($\beta_2$); and C. Fraser, National Institute on Alcohol Abuse and Alcoholism ($\gamma_{2L}$).

GABA currents were measured with a Warner OC725 two-electrode voltage-clamp amplifier 2-5 days following 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 co-applied 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 *Xenopus laevis* tadpoles and in BALB/c mice were performed as previously described (Covey et al., 2000). Briefly, groups of 10 early prelim-bud stage *Xenopus laevis* tadpoles (Nasco. Fort Atkinison, 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 were assessed for the loss-of-righting reflex (LRR) and loss-of-swimming (LSR) reflex 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 Sigma Plot version 8.0. For the mouse assay, BALB/C mice were injected i.v. through a tail vein with various doses of 3α5β-18-norACN in an 8% ethanol, 16% cremophor EL (Sigma Chemical Co.) 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 analogues used in this study are shown in Figure 1. The structural variables were the 5α- vs. 5β- 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 $[^{35}\text{S}]$TBPS binding in rat brain membranes: The effects of neurosteroids on $[^{35}\text{S}]$TBPS binding were examined in the presence of 5 µM GABA (Figure 2A and 2B) and in membranes depleted of GABA (Figure 2A and 2B). Since complete removal of GABA is difficult, experiments were also done examining the effects of one neurosteroid, 3α5αACN, in the presence of GABAzine, a competitive GABA antagonist (Figure 2A).

3α5αACN inhibited $[^{35}\text{S}]$TBPS binding, in the presence of 5 µM GABA, with an IC$_{50}$ 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, 3α5αACN showed two-component behavior with enhancement of $[^{35}\text{S}]$TBPS binding at low concentration (EC$_{50}$ = 40 ± 35 nM) and inhibition of binding at higher concentrations (IC$_{50}$ = 1.4 ± 0.4 µM) (Figure 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 $[^{35}\text{S}]$TBPS binding, but also remarkably reduced baseline $[^{35}\text{S}]$TBPS binding (Figure 2B). In rat brain membranes, 5 µM GABA reduced TBPS binding (in the absence of steroid) by amounts varying from 50-85%. In the presence of GABAzine, baseline TBPS binding was reduced (presumably by antagonizing small amounts of residual GABA) but both the enhancing (EC$_{50}$ = 50 ± 22 nM) and inhibitory effects (IC$_{50}$ = 8 ± 2 µM) of 3α5αACN on $[^{35}\text{S}]$TBPS binding were observed (Figure 2A). Of note, the IC$_{50}$ (NS2 effect) value for 3α5αACN was shifted to the right in the presence of GABAzine.

3α5βACN also inhibited $[^{35}\text{S}]$TBPS binding, in the presence of GABA, with an IC$_{50}$ of 63 ± 7 nM and a Hill slope of 0.9. In the absence of GABA, 3α5βACN enhanced $[^{35}\text{S}]$TBPS binding at low concentrations (EC$_{50}$ = 29 ± 36 nM) and partially inhibited at higher concentrations (IC$_{50}$ = 10.7 ± 3.6 µM) (Figure 2C). The endogenous neurosteroids (acetyl at C17) exhibited behavior that was qualitatively
similar to the carbonitrile series; in the absence of GABA, $3\alpha 5\alpha P$ stimulated $[^{35}\text{S}]\text{TBPS}$ binding with an $EC_{50}$ of $54 \pm 39 \text{ nM}$ and inhibited with an $IC_{50} = 3.3 \pm 0.7 \text{ } \mu\text{M}$; $3\alpha 5\beta P$ had an $EC_{50} = 8 \pm 4 \text{ nM}$ and an $IC_{50} = 38.2 \pm 10.7 \mu\text{M}$ (data not shown in the figure).

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

**Interactions of $3\alpha 5\beta-18$-norACN and $3\alpha 5\alpha ACN$ at the NS1 and NS2 sites:** To determine whether the minimal effect of $3\alpha 5\beta-18$-norACN on NS2 results from poor binding or low efficacy, we examined the interaction of $3\alpha 5\beta-18$-norACN and $3\alpha 5\alpha ACN$. In Figure 3A, the experiments were conducted simultaneously using the same membrane preparation and the same radioligand stock to allow comparison of the absolute amount (fmol/mg protein) of binding. The effect of $3\alpha 5\alpha ACN$ (in the absence of GABA) on $[^{35}\text{S}]\text{TBPS}$ binding was examined in the presence of $3\alpha 5\beta-18$-norACN. $3\alpha 5\beta-18$-norACN (3 µM), a concentration that provides maximum $3\alpha 5\beta-18$-norACN effect (Figure 2D), occluded the enhancing action of $3\alpha 5\alpha ACN$ at NS1, maximally enhancing $[^{35}\text{S}]\text{TBPS}$ binding and preventing any further enhancement by $3\alpha 5\alpha ACN$ (Figure 3A). This indicates that $3\alpha 5\beta-18$-norACN is a full agonist at the NS1 site. The presence of 3 µM $3\alpha 5\beta-18$-norACN also produced a modest change in the $IC_{50}$ value of $3\alpha 5\alpha ACN$ at NS2 (1.4 ± 0.4 µM without $3\alpha 5\beta-18$-norACN and 5.8 ± 4.9 µM in the presence of $3\alpha 5\beta-18$-norACN). To further probe the actions of $3\alpha 5\beta-18$-norACN at NS2, the inhibitory effects of $3\alpha 5\alpha ACN$
Effects of neuroactive steroids on $^{35}$S]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 $^{35}$S]TBPS binding was examined in cell membranes expressing defined combinations of GABA$_A$ receptor subunits. These studies employed $\alpha_1\beta_2$ heteropentamers. Based on the poor ability of $\beta_2$ 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 $\alpha_1\beta_2$ GABA$_A$ receptor subunits were modulated by $3\alpha_5\alpha$ACN in a manner very similar to that observed in rat brain membranes. In the absence of GABA, $3\alpha_5\alpha$ACN stimulated TBPS binding at low concentrations ($\text{EC}_{50}=28 \pm 14 \text{nM}$) and inhibited at higher concentrations ($\text{IC}_{50}=537 \pm 115 \text{nM}$). In the presence of GABA (1 $\mu$M), $3\alpha_5\alpha$ACN inhibited TBPS binding with an $\text{IC}_{50}$ of 20 $\pm$ 9 nM and a Hill slope =1 (Figure 4A). $3\alpha_5\beta$-18-norACN appeared to be a selective NS1 agonist in $\alpha_1\beta_2$ receptors (Figure 4B): In the absence of GABA, $3\alpha_5\beta$-18-norACN enhanced $^{35}$S]TBPS binding with an $\text{EC}_{50}$ of 50 $\pm$ 16 nM and in the presence of 1 $\mu$M GABA it partially (65% inhibition) inhibited TBPS binding with an $\text{IC}_{50}$ of 20 $\pm$ 9 nM. We also examined the concentration-dependent effects of GABA on $^{35}$S]TBPS binding in QT-6 cells expressing $\alpha_1\beta_2$ GABA$_A$ receptor subunits. Consistent with previous studies (Pregenzer et al., 1993) GABA enhances TBPS binding at low concentrations and inhibits at higher concentrations (Figures 4C).
Structural requirements for steroids providing low efficacy at the NS2 site: To determine which structural properties of 3α5β-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 Figure 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 (minimum 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 5β-reduced steroids than in the 5α-reduced steroids. Absence of the 19-methyl group affected neither minimum binding nor the Hill slope. In the C17-acetyl series (Table 2), a similar effect of the 18-nor and 5β-reduced configurations was observed. Of note, pregnanolone (3α5βP) 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 5β-reduced configuration both contribute to lack of neurosteroid efficacy at the NS2 site.

Electrophysiological effects of 3α5β-18-norACN: The ability of 3α5β-18-norACN, 3α5αACN and 3α5βACN to potentiate GABA-elicited (2 μM) currents and to directly activate GABA A receptors was examined in Xenopus Laevis oocytes expressing α1β2γ2L GABA A 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 3α5β-18-norACN, 3α5βACN, or 3α5αACN. The concentration-response curves (Figure 5B) demonstrate that 3α5αACN (Emax= 14± 21) has modestly higher efficacy than 3α5β-18-norACN (Emax= 10 ± 0.3) or 3α5βACN (Emax= 7.8 ± 1.6) in potentiating GABA-elicited currents. However, there is no statistical difference among them. Two-way ANOVA 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 potentiation of 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. In order 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 Figure 6B, 3α5β-18-norACN elicited very small GABA currents compared to 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 (Figure 6B, lower panel), the observed EC$_{50}$ of 3α5β-18-norACN for direct gating was 1.6 ± 0.4 μM. 30 μM 3α5βACN elicited currents equal to 5.0 ± 0.2 % of the 2 μM GABA currents, with an EC$_{50}$ 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$_{50}$ of 3α5αACN could not be accurately determined since maximum 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 μA and 0.03 ± 0.01 μA, respectively (Figure 6C). 30 μM 3α5β-18-norACN 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 (Figure 6D, ***, p<0.001 vs. 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, loss-of-righting reflex (LRR) and loss-of-swimming reflex (LSR) were examined in *Xenopus laevis* pre-limb-bud stage tadpoles exposed to varying concentrations of 3α5β-18-norACN. 3α5β-18-norACN caused LRR with an EC₅₀ value of 164 ± 40 nM (Figure 7A). Our previous work showed that the EC₅₀ values for LRR by 3α5αACN, 3α5αP, 3α5βACN, and 3α5βP in tadpoles were 70 ± 10 nM, 390 ± 40 nM, 80 ± 13nM, and 61± 4 nM, respectively (Covey et al., 2000; Wittmer et al., 1996). They are not statistically significantly different compared to 3α5β-18-norACN. 1.0 µM 3α5β-18-norACN caused no LSR. However, 3 and 10 µM 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 (Figure 7B).

**DISCUSSION**

This paper 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 GABAₐ 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 GABAₐ receptor currents; it appears to be a weak partial agonist at the site mediating direct activation of GABAₐ receptors, as it reduces the direct activation of GABAₐ currents elicited by 3α5αACN.
The action of neuroactive steroids on TBPS binding. [35S]TBPS (a cage convulsant that binds at the picrotoxin site on GABA\(_A\) receptors) binding is a useful reporter for the actions of allosteric modulators of GABA\(_A\) receptors. In well-washed brain membranes and in recombinant GABA\(_A\) receptors, 3\(\alpha 5\alpha\)ACN enhances [35S]TBPS binding at low concentrations and inhibits it at higher concentrations (Figure 2 and Figure 4), consistent with previous observations of similar actions of allopregnanolone ((3\(\alpha,5\alpha\))-3-hydroxypregnan-20-one), pregnanolone 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; Akk et al., 2009; Braccamontes and Steinbach, 2009). Sites NS1 and NS2 do not represent steroid binding sites on distinct GABA\(_A\) receptors differing in subunit composition, since both sites are observed in recombinant \(\alpha_1\beta_2\) GABA\(_A\) receptors (Davies et al., 1997) (Figure 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 (Figure 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 are not addressed in this model. The stoichiometry of TBPS (picrotoxin) binding to GABA\(_A\) receptors is not precisely known. While there may be multiple NS1 and/or NS2 sites on a pentameric GABA\(_A\) 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 mono-ligated receptors will predominate, whereas di-ligated receptors will be the principal species at high GABA concentrations. The above 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 \[^{[35]S}\]TBPS (Figure 8). When the receptor is mono-liganded with GABA (RG) or site NS1 is occupied (RS\(_1\)), it’s affinity for TBPS is increased, resulting in increased \[^{[35]S}\]TBPS binding (See Figure 2A, 4A, 4C) (Luddens and Korpi, 1995; Pregenzer et al., 1993). For neurosteroids, the NS1-mediated increase in TBPS binding results from an increase in the receptor’s affinity for TBPS, since TBPS binding curves performed in the presence of low concentrations of ACN (100 nM) demonstrate a lower K\(_d\) without an increase in B\(_{\text{max}}\) when compared to binding curves performed in the absence of neurosteroid (data not shown). When the receptor is mono-liganded with GABA and site NS1 is occupied (RS\(_1\)G), \[^{[35]S}\]TBPS binding is partially inhibited. This partial inhibition is most apparent when \(3\alpha5\beta\)-18-norACN occupies NS1, since it has minimal efficacy at NS2 (See Figures 2D and 4B). Finally, when the receptor is either di-liganded with GABA (RGG) or both NS1 and NS2 are occupied (RS1S2), the receptors are unable to bind \[^{[35]S}\]TBPS.

**Action of \(3\alpha5\beta\)-18-norACN on TBPS binding.** \(3\alpha5\beta\)-18-norACN behaves as a selective NS1 site agonist. In the absence of GABA (RS1 in Figure 8), it enhances TBPS binding at low concentrations and minimally inhibits TBPS binding at higher concentrations (Figure 2D). \(3\alpha5\beta\)-18-norACN also occludes the NS1 actions of \(3\alpha5\alpha\)ACN, indicating that these two ligands compete for binding at site NS1 and have similar efficacy (Figure 3A). In contrast, while \(3\alpha5\beta\)-18-norACN alone produces no NS2 site effect, increasing concentrations of \(3\alpha5\beta\)-18-norACN antagonize the actions of \(3\alpha5\alpha\)ACN as an NS2 site agonist (Figure 3B); this suggests that \(3\alpha5\beta\)-18-norACN occupies the NS2 site but has minimal efficacy.

In the presence of 5 \(\mu\)M GABA (Figure 2), both the NS1 and NS2 sites contribute to complete inhibition of TBPS binding. \(3\alpha5\beta\)-18-norACN only partially inhibits TBPS binding indicating that it lacks the NS2
site effect (Figures 2D and 4B; RS1G in Figure 8). An 18-methyl group and a 5α-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 non-identical and indicate the feasibility of developing selective agonists and antagonists for these distinct steroid binding sites. A number of other steroid analogues with the 5β-reduced configuration, including the 3,20-pregnadiols and (3α,5β)-3,21-dihydroxypregnan-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 Gee, 1989; Hawkinson et al., 1996; Morrow et al., 1990; Xue et al., 1997). These compounds may also be selective ligands for either the NS1 or NS2 sites.

Electrophysiological action of 3α5β-18-norACN. At a macroscopic level, 3α5β-18-norACN potentiates GABA-elicited currents with potency and efficacy similar to that of 3α5αACN (Figure 5). 3α5β-18-norACN shows minimal efficacy as a direct activator of GABA_δ receptors (α1β2γ2L) expressed in *Xenopus* oocytes, (Figure 6). Furthermore, 3α5β-18-norACN antagonizes the direct activation elicited by 3α5αACN, suggesting that it is a weak partial agonist at the site mediating direct activation of GABA_δ 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_δ 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_δ receptor at high concentrations. Site-directed mutagenesis studies indicate that these electrophysiological effects are mediated by two neurosteroid
binding sites, one that mediates potentiation and one that mediates direct activation (Hosie et al., 2006). While 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-elicted 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 currents. Finally, 3αβ-18-norACN prevents the actions of 3αα-ACN as an NS2 agonist in TBPS binding and as a direct activator of GABA currents. While these data support the hypothesis that the NS1 and NS2 sites are synonymous with the potentiating and direct activating sites, more extensive studies will be 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 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 αβγ-subunit GABA receptors (Akk et al., 2004; Li et al., 2007) Their studies were performed with 50 μM GABA, a concentration at which most receptors are di-liganded 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 di-liganded 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 3α5β-18-norACN. Compound 3α5β-18-norACN also provides a tool for understanding the biological actions of neurosteroids at the NS1 and NS2 sites. 3α5β-18-norACN produces loss-of-righting reflex in *Xenopus* tadpoles and in mice (Figure 7). The loss-of-righting reflex and loss-of-swimming reflex effects of 3α5β-18-norACN have a similar concentration dependence to those of 3α5αACN and 3α5βACN (Covey et al., 2000; Wittmer et al., 1996). Since 3α5αACN, 3α5β-ACN and 3α5β-18-norACN all have similar efficacy at NS1 and as potentiators of GABA-elicited currents and 3α5β-18-norACN has minimal efficacy at NS2 or as a direct activator of GABA<sub>A</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 3α5β-18-norACN in radioligand binding and electrophysiological assays increases the evidence that there are two classes of neurosteroid binding sites on GABA<sub>A</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 vital tool for elucidating the biological actions of endogenous neurosteroids and may provide useful clinical agents.

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Xue BG, Whittemore ER, Park CH, Woodward RM, Lan NC and Gee KW (1997) Partial agonism by 3\( \alpha,21\)-dihydroxy-5 \( \beta \)-pregnan-20-one at the \( \gamma \)-aminobutyric acidA receptor neurosteroid site.1. *J Pharmacol Exp Ther* **28(1)**:1095-1101.
FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Structures of two series of neurosteroid analogues based on either a 5α-reduced or a 5β-reduced steroid backbone. Structural variables include methyl groups at C18 (R₂) and C19 (R₁) and acetyl vs. carbonitrile groups at C17 (R₃).

Figure 2. Effects of neuroactive steroids on specific [³⁵S]TBPS binding to rat brain membranes in the presence and absence of exogenous GABA. 3α5αACN (Panel A) and 3α5βACN (Panel C) modulate [³⁵S]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 GABAzone (Panel A) both the stimulatory and inhibitory effects of 3α5αACN are observed. Panel B: The same data as in panel A plotted as fmol of specific [³⁵S]TBPS binding per mg of membrane protein. Panel D: Compound 3α5β-18-norACN is a preferential NS1 site agonist. In the absence of GABA, 3α5β-18-norACN selectively enhances [³⁵S]TBPS binding with minimal inhibition at higher concentrations. 3α5β-18-norACN partially inhibits TBPS binding in the presence of 5 μM GABA. Data shown are the means of triplicate determinations for representative experiments.

Figure 3. 3α5β-18-norACN occludes enhancement and antagonizes inhibition of [³⁵S]TBPS binding by 3α5αACN in GABA-depleted rat brain membranes. A. [³⁵S]TBPS binding (fmol/mg protein) data demonstrates that 3 μM 3α5β-18-norACN occludes the enhancement action of 3α5αACN at site NS1 and modestly right-shifts its inhibitory effect at site NS2. B. 3α5β-18-norACN antagonizes the inhibitory effect of 10 μM 3α5αACN on [³⁵S]TBPS binding in a concentration-dependent manner.

Figure 4. Effects of 3α5αACN, 3α5β-18-norACN and GABA on [³⁵S]TBPS binding to α₁β₂ GABAₐ receptors expressed in QT-6 cells. A. In the absence of added GABA, 3α5αACN modulates [³⁵S]TBPS
binding to $\alpha_{1\text{myc}}\beta_2\text{FLAG}$ receptors in a biphasic manner ($EC_{50} = 28 \pm 14 \text{nM}$; $IC_{50} = 537 \pm 115 \text{nM}$). In the presence of 1 $\mu$M GABA, enhancement is eliminated and $3\alpha5\alpha\text{ACN}$ only inhibits TBPS binding ($IC_{50} = 20 \pm 9 \text{nM}$). B. In the absence of GABA, $3\alpha5\beta18$-norACN selectively enhances $[^{35}\text{S}]$TBPS binding to $\alpha_{1\text{myc}}\beta_2\text{FLAG}$ receptors ($EC_{50} = 50 \pm 16 \text{nM}$). In the presence of 1 $\mu$M GABA $3\alpha5\beta18$-norACN partially inhibits $[^{35}\text{S}]$TBPS binding ($IC_{50} = 20 \pm 9 \text{nM}$). C. GABA modulates $[^{35}\text{S}]$TBPS binding to $\alpha_{1\text{myc}}\beta_2\text{FLAG}$ receptors in a biphasic manner ($EC_{50} = 119 \pm 1 \text{nM}$; $IC_{50} = 120 \pm 1 \text{nM}$).

Figure 5. Neuroactive steroids potentiate GABA currents in Xenopus oocytes expressing $\alpha_1\beta_2\gamma_2\text{L}$ GABA$\_A$ receptor subunits. A. Superimposed traces of representative currents elicited by 2 $\mu$M GABA alone (lowest amplitude trace)) and 2 $\mu$M GABA plus 0.1, 1 or 10 $\mu$M $3\alpha5\beta18$-norACN, $3\alpha5\beta\text{ACN}$, or $3\alpha5\alpha\text{ACN}$. B. The concentration-response curves for $3\alpha5\beta18$-norACN, $3\alpha5\beta\text{ACN}$, and $3\alpha5\alpha\text{ACN}$ indicate that these three neuroactive steroids have similar potency and efficacy as potentiators of GABA-elicited currents.

Figure 6. Neurosteroids directly activate GABA$\_A$ receptors in Xenopus oocytes expressing $\alpha_1\beta_2\gamma_2\text{L}$ GABA$\_A$ receptor subunits. A. Superimposed traces of representative currents elicited by 10 $\mu$M $3\alpha5\beta18$-norACN, $3\alpha5\beta\text{ACN}$, and $3\alpha5\alpha\text{ACN}$. B. Concentration-response curves for direct activation of the GABA$\_A$ receptors by $3\alpha5\beta18$-norACN, $3\alpha5\beta\text{ACN}$, and $3\alpha5\alpha\text{ACN}$. Curves for $3\alpha5\beta18$-norACN and $3\alpha5\beta\text{ACN}$ are enlarged in the lower panel with $EC_{50}$ equal to 1.6 $\pm$ 0.4 $\mu$M and $3.3 \pm 0.2 \mu$M respectively. $3\alpha5\alpha\text{ACN}$ has significantly higher maximum gating than $3\alpha5\beta\text{ACN}$ or $3\alpha5\beta18$-norACN ($p<0.001$); but there is no significant difference between $3\alpha5\beta\text{ACN}$ $3\alpha5\beta18$-norACN ($p>0.05$, n = 4 oocytes tested at each concentration). C. Representative GABA$\_A$ receptor activation traces elicited by 10 $\mu$M $3\alpha5\alpha\text{ACN}$, 30 $\mu$M $3\alpha5\beta18$-norACN, or a mixture of 10 $\mu$M $3\alpha5\alpha\text{ACN}$ with 30 $\mu$M $3\alpha5\beta18$-norACN. D. 30 $\mu$M...
3α5β-18-norACN significantly antagonizes the direct activation of GABA$_A$ receptors by 10 μM 3α5αACN (***, p<0.001, n=4).

**Figure 7. Anesthetic effects of 3α5β-18-norACN in *Xenopus laevis* tadpoles and BALB/c mice. A.** Compound 3α5β-18-norACN caused loss of righting reflex (LRR) and loss of swimming reflex (LSR) in tadpoles. Points on the tadpole concentration-response curves represent 10-20 animals, scored quantally. The EC$_{50}$ for LRR was 164 ± 40 nM (S.E.). 0.3 μM 3α5β-18-norACN 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.

**Figure 8. Model of neurosteroid and GABA modulation of [$^{35}$S]TBPS binding.** In the absence of bound GABA or neurosteroid, the receptor (R) can bind [$^{35}$S]TBPS. When the receptor is mono-liganded with GABA (RG) or site NS1 is occupied (RS$_1$), its affinity for TBPS is increased, resulting in increased [$^{35}$S]TBPS binding. In receptors that are mono-liganded with both GABA and S1 (RS$_1$G), TBPS binding is partially inhibited. This partial inhibition is most apparent when 3α5β-18-norACN occupies site NS1, since it has minimal efficacy at site NS2. When receptors are bi-liganded at either both GABA sites (RGG) or both steroid sites (RS$_1$S$_2$), TBPS binding is completely inhibited.
Table 1: C17-carbonitrile neuroactive steroids: modulation of [35S]TBPS binding in rat brain membranes. Concentration-response curves were generated for the inhibition of specific [35S]TBPS binding in the presence of 5 μM GABA. Curves were fit to a single-component Hill equation; Hill coefficients, IC₅₀ values (mean ± SD of triplicate determinations) and minimum binding are reported for all compounds.

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<tr>
<th>Compound</th>
<th>Hill Slope</th>
<th>IC₅₀ (nM)</th>
<th>Min Binding (%)</th>
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<tr>
<td>3α5β-18-norACN</td>
<td>1.27</td>
<td>49 ± 4</td>
<td>41 ± 1</td>
</tr>
<tr>
<td>3α5β-19-norACN</td>
<td>0.82</td>
<td>22 ± 3</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>3α5β-18,19-dinorACN</td>
<td>1.05</td>
<td>73 ± 16</td>
<td>31 ± 3</td>
</tr>
</tbody>
</table>
### TABLE 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Hill Slope</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>Min Binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3α5αP</td>
<td>0.94</td>
<td>69 ± 8</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>3α5α-18-norP</td>
<td>0.90</td>
<td>157 ± 16</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>3α5α-19-norP</td>
<td>0.99</td>
<td>86 ± 7</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>3α5βP</td>
<td>0.65</td>
<td>51 ± 16</td>
<td>7 ± 5</td>
</tr>
<tr>
<td>3α5β-18-norP</td>
<td>0.85</td>
<td>35 ± 11</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>3α5β-19-norP</td>
<td>1.01</td>
<td>12 ± 1</td>
<td>9 ± 1</td>
</tr>
</tbody>
</table>

Table 2: C17-acetyl neuroactive steroids: modulation of [<sup>35</sup>S]TBPS binding in rat brain membranes. Concentration-response curves were generated for the inhibition of specific [<sup>35</sup>S]TBPS binding in the presence of 5 μM GABA. Curves were fit to a single-component Hill equation; Hill coefficients, IC<sub>50</sub> values (mean ± SD of triplicate determinations), and minimum binding are reported for all compounds.
Figure 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>3α,5α-P</td>
<td>Me</td>
<td>Me</td>
<td>Acetyl</td>
</tr>
<tr>
<td>3α,5α-ACN</td>
<td>Me</td>
<td>Me</td>
<td>CN</td>
</tr>
<tr>
<td>3α,5α-18-norP</td>
<td>Me</td>
<td>H</td>
<td>Acetyl</td>
</tr>
<tr>
<td>3α,5α-18-norACN</td>
<td>Me</td>
<td>H</td>
<td>CN</td>
</tr>
<tr>
<td>3α,5α-19-norP</td>
<td>H</td>
<td>Me</td>
<td>Acetyl</td>
</tr>
<tr>
<td>3α,5α-19-norACN</td>
<td>H</td>
<td>Me</td>
<td>CN</td>
</tr>
<tr>
<td>3α,5α-18,19-dinorACN</td>
<td>H</td>
<td>H</td>
<td>CN</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>3α,6β-P</td>
<td>Me</td>
<td>Me</td>
<td>Acetyl</td>
</tr>
<tr>
<td>3α,6β-ACN</td>
<td>Me</td>
<td>Me</td>
<td>CN</td>
</tr>
<tr>
<td>3α,6β-18-norP</td>
<td>Me</td>
<td>H</td>
<td>Acetyl</td>
</tr>
<tr>
<td>3α,6β-18-norACN</td>
<td>Me</td>
<td>H</td>
<td>CN</td>
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<tr>
<td>3α,6β-19-norP</td>
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<td>Me</td>
<td>Acetyl</td>
</tr>
<tr>
<td>3α,6β-19-norACN</td>
<td>H</td>
<td>Me</td>
<td>CN</td>
</tr>
<tr>
<td>3α,6β-18,19-dinorACN</td>
<td>H</td>
<td>H</td>
<td>CN</td>
</tr>
</tbody>
</table>
Figure 2
Figure 3

A.

B.
Figure 4

A

Specific $[^{35}S]$,TBPS Binding (% of control)

$[3\alpha 5\alpha ACN](M)$

B

Specific $[^{35}S]$,TBPS Binding (% of control)

$[3\alpha 5\beta-18-norACN](M)$

C

Specific $[^{35}S]$,TBPS Binding (% of control)

$[GABA](M)$
Figure 5

A. $3\alpha_5\beta$-18-norACN

- 2 µM GABA
- +0.1 µM
- +1 µM
- +10 µM

B. Fold to 2 µM GABA

- $3\alpha_5\beta$-18-norACN
- $3\alpha_5\beta$ACN
- $3\alpha_5\alpha$ACN

[Graph showing the fold to 2 µM GABA for different neurosteroids]
Figure 7
Figure 8