British Journal of Pharmacology (2005) 146, 894–902
www.nature.com/bjp

Low concentrations of neuroactive steroids alter kinetics of [^{3}H]ifenprodil binding to the NMDA receptor in rat frontal cortex

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1 The modulatory effects of the two neurosteroids pregnenolone sulphate (PS) and pregnanolone sulphate (3α5βS) on [^{3}H]ifenprodil binding to the *N*-methyl-D-aspartate (NMDA) receptor in rat frontal cortex were studied.

2 The binding for [^{3}H]ifenprodil itself displayed monophasic kinetics in all experiments. None of the neurosteroids displaced the radioligand from its binding site on the NR2B subunit of the NMDA receptor. However, their continual presence at nanomolar concentrations had significant effects on ligand binding kinetics, interacting through distinct sites in saturation, competition and dissociation experiments.

3 PS at 30 nM enhanced the specific binding to about 150% of that in its absence and enhanced the dissociation rate three-fold indicating a positive modulation of [^{3}H]ifenprodil binding to the NMDA receptor. Furthermore, PS increased B_{max} and decreased K_d suggesting that the neurosteroid exposes new [^{3}H]ifenprodil binding sites with altered properties.

4 In contrast, 3α5βS (30 nM) decreased specific [^{3}H]ifenprodil binding to approximately 40% of that determined for the radioligand alone. The presence of 3α5βS at nanomolar concentrations induced biphasic curve fits in saturation, competition as well as dissociation experiments.

5 In conclusion, the present study show that the allosteric modulators PS or 3α5βS change [^{3}H]ifenprodil binding kinetics in a way indicating conformational alteration of its binding site on the NR2B subunit.

British Journal of Pharmacoogy (2005) **146,** 894–902. doi:10.1038/sj.bjp.0706397;
published online 19 September 2005

Keywords: NMDA receptor; NR2B subunit; neurosteroid; ifenprodil; receptor binding; allosteric modulation

Abbreviations: PS, pregnenolone sulphate; 3α5βS, pregnanolone sulphate

Introduction

The endogenous neuroactive steroids pregnenolone sulphate (PS) and pregnanolone sulphate (3α5βS) can rapidly alter neuronal excitability by interaction with different membrane receptors, such as the $GABA_A$ and the *N*-methyl-D-aspartate (NMDA) receptor (for reviews see Baulieu, 1998; Compagnone & Mellon, 2000). Their effects are believed to be mediated by nongenomic mechanisms and include allosteric modulation.

At the NMDA receptor PS typically acts as a positive modulator while 3α5βS is inhibiting. PS has been shown to protect cultured cells from NMDA excitotoxicity and mice from NMDA-induced seizures (Weaver *et al*., 1997). In mice and rats it is shown to enhance memory and cognitive performances (Flood *et al*., 1992; Meziane *et al*., 1996) and reverse NMDA antagonist-induced memory deficits (Mathis *et al*., 1994; 1996). PS potentiates NMDA-activated whole-cell currents (Wu *et al*., 1991; Bowlby, 1993), while 3α5βS inhibits such currents (Park-Chung *et al*., 1994; 1997; Baulieu, 1998). In electrophysiological studies on recombinant NMDA receptors PS has been shown to act as a positive modulator on receptors composed of NR1/NR2A and NR1/NR2B subunit forms while it inhibits NR1/NR2C and NR1/NR2D forms (Malayev *et al*., 2002). In the same study 3α5βS was found to act as an inhibitory modulator on all four subtypes. PS also stimulates Ca^{2+} signalling through NR1/NR2B channels (Mukai *et al*., 2000). The neurosteroid binding sites have been shown to be distinct from those for spermine, glycine, phencyclidine, arachidonic acid, Mg^{2+} and protons (Park-Chung *et al*., 1997) and in a recent study it was suggested that a steroid modulatory domain located on the NR2B subunit could be responsible for the allosteric interaction caused by PS (Jang *et al*., 2004).

Ifenprodil, a heterocyclic amino alcohol, is an atypical noncompetitive antagonist, which selectively binds to and inhibits NR2B-containing NMDA receptors (Carter *et al*., 1990; Mott *et al*., 1998; Williams, 2001). Owing to its subunit selectivity [^{3}H]ifenprodil has been extensively used as a ligand in receptor-binding studies on NR2B-containing NMDA receptor complexes (Chenard & Menniti, 1999). Recently, we demonstrated that nanomolar concentrations of dehydroepiandrosterone sulphate (DHEAS) and allopregnanolone sulphate (ALLOPREGS) could alter the kinetics of [^{3}H]ifenprodil binding to rat cortical membranes, a rich source for

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NR2B-containing NMDA receptors (Johansson & Le Greves, 2005). In this study, we investigated and characterised the effect of low amounts of PS or 3α5βS using the same approach. As mentioned above, the modulatory effects of PS or 3α5βS on the NMDA receptor have been characterised by use of electrophysiological techniques, although it requires relatively high concentrations of the steroids. Here, we show that more than 1000-fold lower concentrations of PS and 3α5βS differentially modulate the binding characteristics for [3H]ifenprodil to the NMDA receptor by acting through distinct sites.

Methods

Membrane preparation

The animal experimental procedure was approved by the local experimental animal committee. Adult male Sprague–Dawley rats (Alab AB, Sollentuna, Sweden) weighing 200–220 g (approximately 11-week-old) were killed and the brain tissues were rapidly dissected out on ice and placed on dry ice. Tissues were kept at −80°C until further processed. Frontal cortex from 18 animals were pooled (total weight 1.27 g) and homogenised in 10 volumes of ice-cold 0.32 M sucrose/50 mM Tris/acetate buffer (pH 7.4) (homogenising buffer) in a Teflon pestle homogeniser. The homogenate was centrifuged at $1000 \times g$ for 10 min at 4°C and the supernatant was collected. The pellet was dissolved in a small volume of homogenising buffer and centrifuged again at $1000 \times g$ for 10 min at 4°C. The supernatant was collected together with the first supernatant and centrifuged at $17{,}000 \times g$ for 20 min at 4°C. The pellet (P2) was homogenised in 20 volumes of ice-cold 50 mM Tris/acetate buffer, pH 7.0 and centrifuged at $50{,}000 \times g$ for 30 min at 4°C. This last centrifugation step was repeated three times. The final pellet was suspended in ice-cold 50 mM Tris/acetate buffer, pH 7.0 and aliquots of 0.5 ml were stored at −80°C. The protein content was measured by the method described by Lowry *et al*. (1951).

[3H]ifenprodil binding assay

Triplicate incubations were carried out in 96-deep wells micro titre plates for 2 h at room temperature (water bath, 25°C), in order to establish equilibrium. The assay was carried out in a total volume of 500 μl in the presence of 0.6 nM [3H]ifenprodil (except for the saturation experiment) in binding buffer (50 mM Tris/acetate buffer, pH 7.0) containing 50 μg of protein and 100 μM of trifluoperazine, in order to block non-NMDA receptor [3H]ifenprodil binding (Coughenour & Barr, 2001). Neurosteroids were dissolved in binding buffer in a stock solution of 100 μM and stepwise diluted to assay concentrations. Unlabelled ifenprodil, 10 μM, was used to define nonspecific binding. The assays were performed in a Biomek 2000 (Beckman Instruments Inc., Fullerton, CA, U.S.A.). Bound radioligand was separated from unbound by filtration (Tomtec 96-well harvester, Tomtec corp., Hamden, CT, U.S.A.) through presoaked (0.3% polyethyleneimine solution) Filtermat B glass fibre filter (Wallac Oy, Turku, Finland), under reduced pressure. The filters were rinsed with 3 ml of cold binding buffer four times. Air was allowed to pass through the filters and they were allowed to dry overnight at room temperature. Solid melt-on MeltiLex B/HS (Wallac Oy, Turku, Finland) was applicated on the dry filters and the radioactivity was measured in a β-counter (Microbeta TriLux, Wallac Oy, Turku, Finland).

Materials

[3H]ifenprodil (67 Ci/mmol) was obtained from Perkin-Elmer Life Science Inc. (Boston, MA, U.S.A.). PS and 3α5βS was purchased from Steraloids (Newport, RI, U.S.A.). AP5 was obtained from Tocris (Bristol, U.K.). The enzyme inhibitor cocktail was purchased from Roche Diagnostics (Bromma, Sweden). Other compounds and reagents were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

Data analysis

All kinetic analysis were calculated and statistically analysed in Prism 4.0 (GraphPad, 2003). Where appropriate, one-way ANOVA, *F*-test or Student's *t*-test (two-tailed) were used for data analysis, considering $P<0.05$ as significant level.

Results

Evaluation of the conditions for membrane preparation and receptor-binding assay

We chose to prepare the cortical membranes in the absence of enzyme inhibitors. To investigate whether this influenced [3H]ifenprodil-specific binding, it was compared to the binding to membranes prepared in the presence of an enzyme inhibitor cocktail. The results showed no significant differences between the two extraction methods. Membranes homogenised without the addition of inhibitors were used throughout the experiments. The specific binding for [3H]ifenprodil alone to the cortical membranes was high and kept within 90–96%. Since we used very low concentrations of the sulphated steroids we wanted to exclude the presence of estrone sulphatases (releasing the unsulphated steroid) during assay incubations. When the estrone sulphatase inhibitor estradiol (100 μM) (Santner & Santen, 1993) was added to the receptor-binding buffer there was no significant difference in PS or 3α5βS modulatory effect on [3H]ifenprodil-specific binding compared to that in the absence of the inhibitor. Furthermore, estradiol alone at 100 μM did not affect [3H]ifenprodil-specific binding.

PS and 3α5βS modulate [3H]ifenprodil-total-specific binding to cortical membranes

To determine whether the neurosteroids affected the specific binding of [3H]ifenprodil to cortical membranes, dose–effect curves were established. Binding assays were run in the absence or presence of PS or 3α5βS at a wide range of concentrations from 10^{-14} to 10^{-5} M. Within the same narrow window (10^{-8}–10^{-7} M) PS enhanced and 3α5βS inhibited [3H]ifenprodil binding in a bell- and U-shape pattern (respectively) as illustrated in Figure 1. Thus, 20, 30 and 100 nM of PS significantly increased [3H]ifenprodil-specific binding to 149±19, 155±18 and 140±23%, respectively, of the vehicle control value (100%), while the same concentrations of 3α5βS significantly decreased the binding 41±13, 39±11 and 52±6%, respectively, of the control value. When the

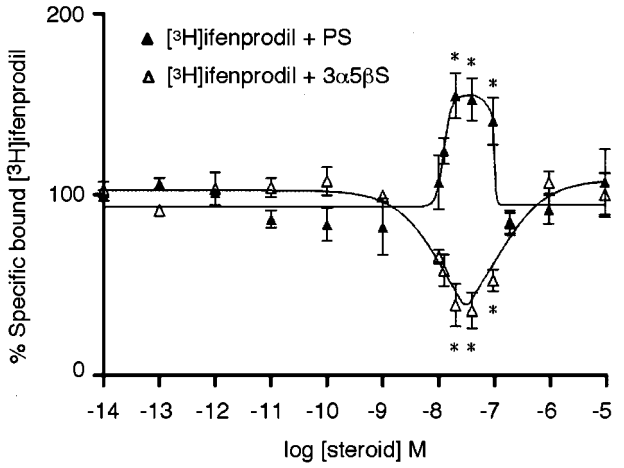

Figure 1 Concentration–effect curves of the continual presence of different concentration of PS or 3α5βS on [3H]ifenprodil-specific binding to the NMDA receptor in rat frontal cortex membranes. Unspecific binding was determined in the presence of 10 μM unlabelled ifenprodil. Binding in the absence of neurosteroid (control) was set to 100%. PS induced a significant enhanced binding at 20, 30 and 100 nM while 3α5βS at the same concentrations significantly reduced the binding of the radioligand. Neurosteroid concentrations yielding maximal modulatory effect (MEC_{max}) and half maximal modulatory effect (MEC_{50}) are given in Table 1. Data points are the mean ± s.e.m. of three separate experiments each analysed in triplicates. Individual data points were connected by a sigmoidal concentration–effect (variable slope) equation. $*P<0.05$ compared to specific binding of [3H]ifenprodil alone (one-way ANOVA followed by Dunnet's *post hoc* test).

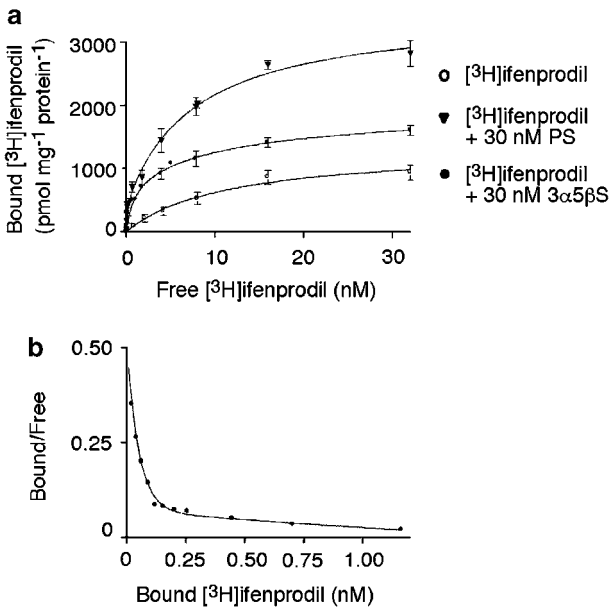

Figure 2 Saturation curves for specific [3H]ifenprodil binding to the NMDA receptor in rat frontal cortex membranes in the absence or presence of PS (30 nM) or 3α5βS (30 nM) (a). The neurosteroid concentrations were chosen from their maximal modulatory effect (MEC_{max}) values. Data were fitted to a monohyperbolic as well as a bihyperbolic equation and statistically compared in a *F*-test. [3H]ifenprodil alone or in combination with PS displayed a monohyperbolic curve fit. However, when 3α5βS was coincubated with [3H]ifenprodil a bihyperbolic curve fit was significantly better compared to the monohyperbolic as illustrated in the Scatchard plot (b). The statistics and values for B_{max} and K_d are presented in Table 2. Each data point represents the mean ± s.e.m. of three separate experiments each analysed in triplicates.

Table 1 Inhibition or enhancement of [3H]ifenprodil binding

Compound	*MEC_{50}* (nM)	*MEC_{max}* (nM)	*% of control*[a]
[3H]ifenprodil + 3α5βS	10.6 ± 2.0[b]	31.6 ± 3.3	30 ± 11
[3H]ifenprodil + PS	12.6 ± 3.4[c]	37.8 ± 4.2	161 ± 18
[3H]ifenprodil + 3α5βS with 30 nM PS present	11.3 ± 2.3[c]	32.5 ± 4.1	115 ± 9
[3H]ifenprodil + PS with 30 nM 3α5βS present	14.1 ± 3.1[c]	39.1 ± 3.9	113 ± 12

The data are mean ± s.e.m. of three separate experiments.
[a]Control (100%) = [3H]ifenprodil alone.
[b]Inhibition.
[c]Stimulation.

Table 2 Saturation of rat frontal cortex homogenate with [3H]ifenprodil

Compound	B_{max} (pmol mg^{-1} protein^{-1})	K_d (nM)
[3H]ifenprodil	1.3 ± 0.12	11.4 ± 2.5
[3H]ifenprodil +		
30 nM 3α5βS, site 1[†]	0.88 ± 0.21	0.90 ± 0.27
30 nM 3α5βS, site 2[†]	1.1 ± 0.12	14.3 ± 3.0
[3H]ifenprodil + 30 nM PS	3.2 ± 0.17**	4.1 ± 0.69**

The data are mean ± s.e.m. of three independent experiments.
[†]Indicate a significant better two-site fit (*F*-test).
$**P<0.01$ (Student's *t*-test, two-tailed).

neurosteroid-induced effects were fitted in the Prism 4.0 program values for the concentration yielding maximal modulatory effect (MEC_{max}) and the concentrations producing 50% of the maximal modulatory effect (MEC_{50}) were calculated (Table 1).

PS and 3α5βS modulate [3H]ifenprodil saturation binding to cortical membranes

Saturation binding experiments were run in the absence or presence of PS or 3α5βS at 30 nM (a concentration close to their MEC_{max}). [3H]ifenprodil was used at concentrations between 0.2 and 32 nM. Data were fitted to a monohyperbolic as well as a bihyperbolic equation and statistically applied to a *F*-test. If the bihyperbolic equation did not statistically improve the curve fit, the monohyperbolic curve fit was chosen. The binding of [3H]ifenprodil alone was saturable and displayed a monophasic curve (Figure 2). In the presence of PS (30 nM) the saturation still displayed a monohyperbolic fit. B_{max} for [3H]ifenprodil was significantly increased while K_d was decreased (Table 2). The addition of 3α5βS (30 nM) resulted in significantly better fit for a bihyperbolic saturation curve for [3H]ifenprodil compared to the monohyperbolic fit ($F=7.5$, $P<0.01$) (Figure 2, Table 2).

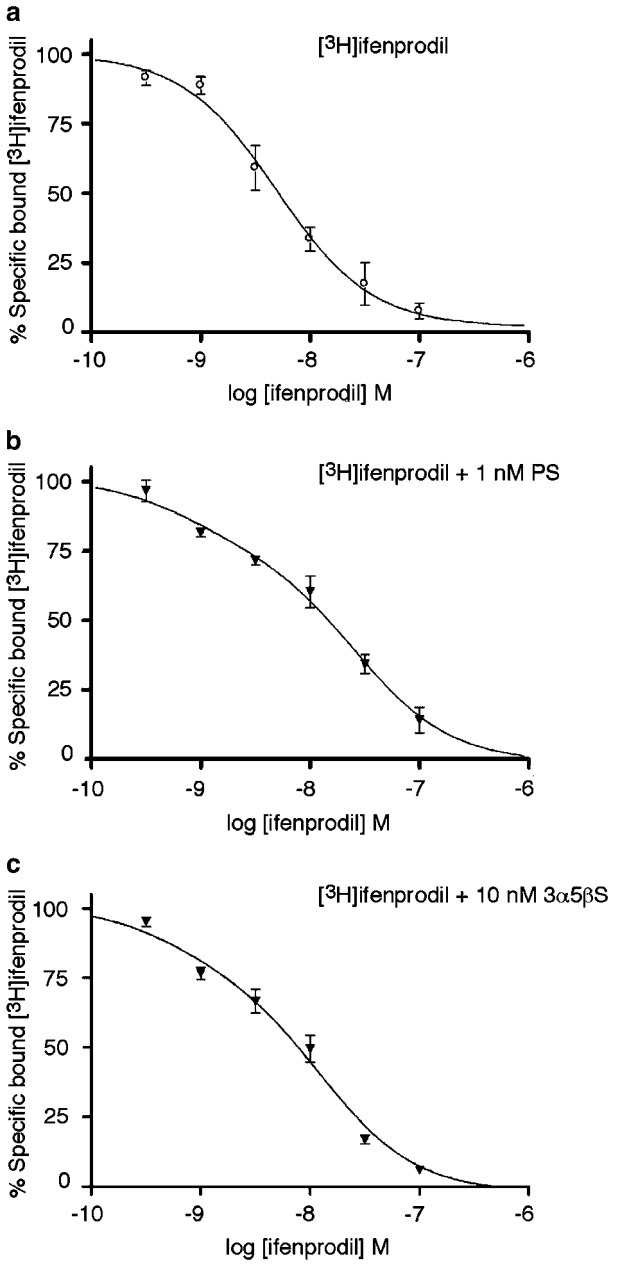

Figure 3 Competition curves of specific-[^{3}H]ifenprodil binding to the NMDA receptor in rat frontal cortex membranes. The binding was inhibited by increasing concentrations of unlabelled ifenprodil alone (a), or in combination with PS (b) or 3α5βS (c) at representative concentrations where the *F*-test indicated a significant better two-site fit competition curve. For PS this effect was seen at concentrations lower compared to those enhancing specific [^{3}H]ifenprodil binding, while 3α5βS induced a two-site fit competition curve at concentrations within or close to those reducing [^{3}H]ifenprodil binding. The values for the [^{3}H]ifenprodil competition curves are given in Table 3. Each data point represents the mean$\pm$s.e.m. of four to six separate experiments each analysed in triplicates.

PS and 3α5βS modulate the displacement of [^{3}H]ifenprodil by ifenprodil

In competition studies, the displacement of [^{3}H]ifenprodil with unlabelled ifenprodil was performed in the absence or presence of PS or 3α5βS at concentrations covering and surrounding their window of effect on [^{3}H]ifenprodil total binding (0.1, 1.0, 10, 100 and 1000 nM). Data were fitted to a one-site as well as two-site equation and compared in a *F*-test. In cases where the two-site equation did not statistically improve the curve fit, the one-site curve fit was chosen. None of the steroids were able to displace [^{3}H]ifenprodil from the cortical membranes (data not shown). The displacement of [^{3}H]ifenprodil with unlabelled ifenprodil was best described using a one-site fit model with a Hill coefficient (n_H) close to unity (0.92) suggesting one single binding site in the absence of neurosteroids (Figure 3, Table 3). The continual presence of PS or 3α5βS at different concentrations had a marked influence on the kinetics of the displacement by unlabelled ifenprodil (Figure 3, Table 3). They were both able to produce biphasic curves for the displacement of [^{3}H]ifenprodil, although at somewhat different concentration interval. PS at 0.1, 1.0, and 10 nM changed the kinetics to a significantly better two-site compared to a one-site fit ($F=3.8$, $P<0.05$; $F=4.2$, $P<0.05$; $F=3.9$, $P<0.05$, respectively) producing a high-affinity fraction of 40–60% across the three different concentrations. PS at concentrations below 0.1 nM or over 10 nM did not have any influence on the curve fit compared to that in its absence; however, the IC_{50} values were significantly increased at PS concentrations of 100 and 1000 nM (Table 3). The two-site fit was also induced in the presence of 3α5βS at 1.0, 10 and 100 nM ($F=21$, $P<0.001$; $F=3.7$, $P<0.05$; $F=58$, $P<0.001$, respectively) with high-affinity fraction between 20 and 25%. At other concentrations the competition binding was best described as monophasic (Table 3).

PS and 3α5βS modulate [^{3}H]ifenprodil dissociation from cortical membranes

Dissociation of [^{3}H]ifenprodil from the NMDA receptor was studied in the presence or absence of 30 nM of either neurosteroid. Dissociation experiment began at equilibrium, reached after 2 h both in the absence or presence of PS or 3α5βS, and was stopped at time points from 0 to 60 min by the addition of unlabelled ifenprodil (100 μM). The dissociation data were fitted to a single as well as a double exponential decay function. In the absence or presence of PS (30 nM) the double exponential decay function did not result in a significant improvement of the curve fit (Figure 4); however, PS had a significant effect on the dissociation rate with a three-fold increase in $t_{1/2}$ (Table 4). The presence of 3α5βS at 30 nM resulted in a significantly better double exponential decay function ($F=8.1$, $P<0.01$) for [^{3}H]ifenprodil, thus revealing a binding site with a high dissociation rate.

PS and 3α5βS act on [^{3}H]ifenprodil-specific binding through distinct sites

To investigate whether PS and 3α5βS act through distinct sites, concentration–effect curves on total specific [^{3}H]ifenprodil binding were performed in the continual presence of PS and 3α5βS in combination (Figure 5). The increase in binding produced by PS (30 nM) was dose dependently inhibited by 3α5βS without any significant effect on MEC_{50} for 3α5βS. Similarly, the presence of 3α5βS (30 nM) did not significantly

Table 3 Displacement of [3H]ifenprodil with unlabelled ifenprodil from rat frontal cortex homogenate

Displacing compound	IC_{50} (nM) *One-site fit*	n_H	$IC_{50\ high}$ (nM) *Two-site fit*	$IC_{50\ low}$	*% High affinity*	n
Ifenprodil	5.0±0.7	−0.92±0.06				6
Ifenprodil + 1 μM PS	7.7±1.1*	−0.91±0.09				4
Ifenprodil + 100 nM PS	13.7±1.8*	−0.84±0.06				6
Ifenprodil + 10 nM PS†	6.0±1.2	−0.61±0.08	0.5±0.2	12.9±2.0	48±12	4
Ifenprodil + 1 nM PS†	12.0±1.6	−0.72±0.10	1.4±0.4	13.4±1.9	43±9	4
Ifenprodil + 0.1 nM PS†	7.8±1.5	−0.80±0.06	1.2±0.4	12.0±1.8	41±10	4
Ifenprodil + 0.01 nM PS	6.9±1.0	−0.95±0.11				4
Ifenprodil + 1 μM 3α5βS	5.4±1.0	−0.92±0.09				4
Ifenprodil + 100 nM 3α5βS†	12.0±1.6	−0.60±0.09	1.4±0.3	58.7±11	26±7	4
Ifenprodil + 10 nM 3α5βS†	8.0±1.2	−0.79±0.07	0.4±0.3	23.7±1.3	26±9	6
Ifenprodil + 1 nM 3α5βS†	2.8±0.9	−0.66±0.10	1.1±0.5	26.7±1.2	21±7	4
Ifenprodil + 0.1 nM 3α5βS	4.4±1.1	−0.91±0.12				4

The data are mean±s.e.m., n = the number of separate experiments.
*$P<0.05$ vs ifenprodil alone (Student's t test, two tailed).
†Indicates a significant better two-site fit (see Results section).

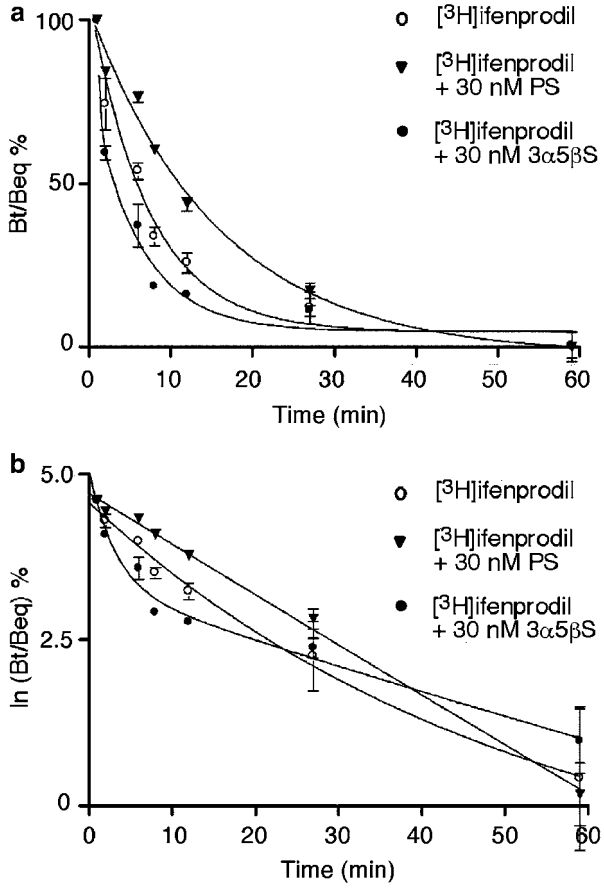

Figure 4 A 60 min time course of the dissociation of [3H]ifenprodil from the NMDA receptor in the presence of 100 μM unlabelled ifenprodil alone, or in combination with either PS (30 nM) or 3α5βS (30 nM) (a). Ifenprodil both alone and in combination with PS produced dissociation kinetics described by a single exponential fit. However, the neurosteroid induced a two-fold enhancement of the dissociation rate (Table 4). The dissociation kinetics in the presence of ifenprodil and 3α5βS described a significantly improved fit by a double exponential equation. This is clearly illustrated by two slopes given when ln ($[B_t]/[B_0]$) (B_t representing radioactivity bound at a given time and B_0 total radioactivity bound at equilibrium) is plotted against elapsed time (b). Each data point represents the mean± s.e.m. of three separate experiments each analysed in triplicates.

Table 4 Dissociation of [3H]ifenprodil from rat frontal cortex homogenate

Compound	k_{off} (min^{-1})	$t_{1/2}$ (min)
[3H]ifenprodil	0.20±0.022	5.0±0.1
[3H]ifenprodil		
+30 nM 3α5βS, site 1†	0.19±0.043	5.3±0.2
+30 nM 3α5βS, site 2†	3.42±1.04	0.29±0.09
[3H]ifenprodil + 30 nM PS	0.064±0.0057*	16±1.7*

k_{off} is the dissociation rate constant for [3H]ifenprodil binding to rat frontal cortex membranes. $t_{1/2}$ is calculated as $1/k_{off}$. The data are mean±s.e.m. of three independent separate experiments.
*$P<0.05$ vs ifenprodil alone (Student's t-test, two-tailed).
†Indicates a significant better two-site fit (F-test).

alter MEC_{50} for PS (Table 1). If the two neurosteroid had competed for the same site, the modulatory effect curve for PS and 3α5βS would have been shifted to the right. The lack of such effects indicates that the two neurosteroids act through distinct sites to modulate the binding of [3H]ifenprodil.

The effect of other NMDA receptor modulatory ligands on PS and 3α5βS action on [3H]ifenprodil-specific binding

To test whether other sites of the NMDA receptor could interact with the action of the neurosteroids, total specific [3H]ifenprodil binding were carried out in the presence of PS or 3α5βS at 30 nM and one of glutamate (100 μM), AP5 (1 mM), glycine (10 μM) or DTT (4 mM). Glutamate enhanced [3H]ifenprodil binding by 22±2.1% ($n=3$). Moreover, glutamate significantly ($P<0.05$) reduced the stimulatory effect of PS on [3H]ifenprodil binding from 160±9.0% in its absence to 139±12% in its presence, whereas the glutamate site antagonist AP5 did not alter PS modulatory effect. There was no effect on PS modulated [3H]ifenprodil binding by glycine or the reducing agent DTT. The negative modulatory effect of 3α5βS on [3H]ifenprodil binding was not significantly modified by any of the substances tested.

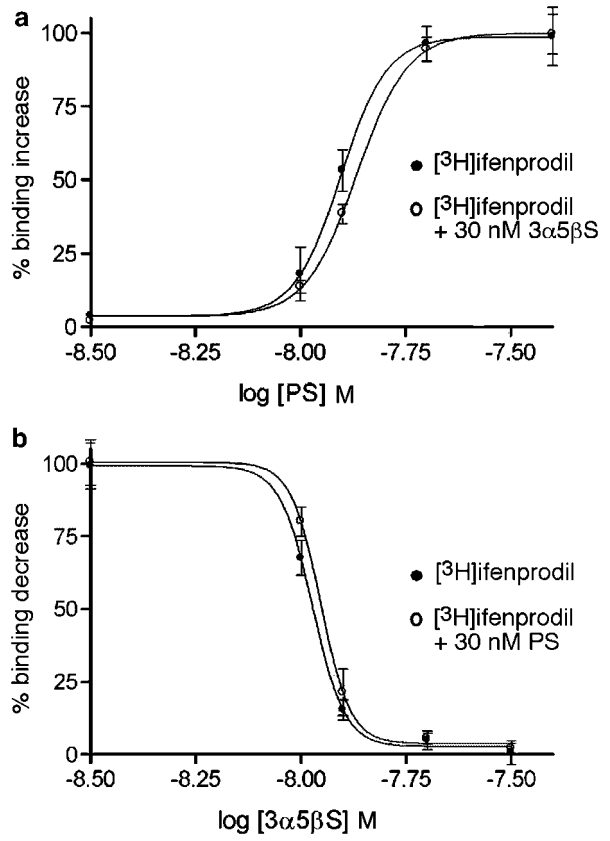

Figure 5 Concentration–effect curves on total specific [^{3}H]ifenprodil binding of (a) PS in the absence or continual presence of 3α5βS (30 nM) and (b) 3α5βS in the absence or continual presence of PS (30 nM). Since 3α5βS or PS did not significantly alter one anothers MEC_{50} values (presented in Table 1), they are suggested to modulate the binding of [^{3}H]ifenprodil through distinct sites and not by a competetive interaction. If the two neurosteroid had competed for the same site, the modulatory effect curve for PS (a) and 3α5βS (b) would have been shifted to the right. Maximal stimulation of [^{3}H]ifenprodil binding by PS is represented by 100% and maximal inhibition by 3α5βS as 0%. Data points are the mean$\pm$s.e.m. of three separate experiments each analysed in triplicates. Individual data points were connected by a sigmoidal dose–response (variable slope) equation.

Discussion

We have in this study utilised [^{3}H]ifenprodil to trace allosteric modulation of NR2B subunit-containing NMDA receptors in cortical membranes isolated from adult rats. Such approach could be argued against since ifenprodil (a); does not bind solely to NMDA receptors (Hashimoto & London, 1993) (b); displays a high- and a low-affinity binding site in ligand binding assays (Reynolds & Miller, 1989), sites which are believed to represent NR1/NR2B and NR1/NR2A composed receptors, respectively (Williams, 1993) and (c); the NR2B subunit in the rat brain is predominantly expressed during development (Monyer *et al*., 1994). However, by the inclusion of trifluoperazine in the binding assay we isolated the binding to the NMDA receptor (Coughenour & Barr, 2001), secondly, by use of low concentrations of [^{3}H]ifenprodil only the high-affinity binding site was revealed as it is known that NMDA receptor complexes in form of NR1/NR2A show a 400-fold lower affinity for ifenprodil compared to the NR1/NR2B channel (Williams, 1993). Finally, despite the developmental switch in subunit composition, the NR1/NR2B form is reported to represent a considerable part of NMDA receptors expressed in the adult mammalian forebrain (Chazot & Stephenson, 1997). Thus, we are convinced that the [^{3}H]ifenprodil binding as determined in this study represents its high-affinity binding site on the NR1/NR2B receptor channel.

The results show that PS and 3α5βS exert differential effects on [^{3}H]ifenprodil binding to adult rat cortical membranes. The neurosteroids themselves did not displace [^{3}H]ifenprodil; however, their continual presence altered the kinetics for the binding of the radioligand with characteristics of an allosteric interaction between the neurosteroids and the binding site for [^{3}H]ifenprodil. The modulatory effects were seen within narrow windows of nanomolar concentrations of PS and 3α5βS suggesting that they act through binding sites with higher affinity for the neurosteroids compared to those mediating the action on NMDA receptors observed in electrophysiological studies.

PS and 3α5βS act as positive or negative modulators, respectively, at the NMDA receptor when responses are studied electrophysiologically. However, this requires relatively high concentrations of the steroids (approximately 100 μM) (Ceccon *et al*., 2001; Malayev *et al*., 2002; Horak *et al*., 2004) contrasting the observed effects of very low doses of PS when injected intracerebroventricular (i.c.v.) in rodents (Flood *et al*., 1992; 1995; Mayo *et al*., 1993; Mathis *et al*., 1994; 1996). The modulatory effects of PS and 3α5βS on [^{3}H]ifenprodil binding as seen in this study also occur at low steroid concentrations (1–100 nM), levels that are possibly more close to what could be considered as physiological. However, it is difficult to estimate the endogenous levels of neurosteroids at the synapse, but in a recently reported study PS concentrations in the rat brain were found to be 5.9 ng/g (Yan & Hou, 2004) while others have estimated it to be less than 0.3 ng/g wet tissue (Liu *et al*., 2003). The mechanism behind the effects of low doses of PS and 3α5βS is unclear and it may be different from that behind the effects seen at much higher concentrations in electrophysiological experiments. It is tempting to speculate that both high- and low-affinity binding sites for neurosteroids exist on the NMDA receptor complex, that is, the effect seen electrophysiologically may be mediated through low-affinity neurosteroid binding sites while the modulatory action on cognitive functions and as seen in this study, the modulation of [^{3}H]ifenprodil binding, are through high-affinity sites.

In order to compare putative high-affinity neurosteroid sites with those identified in functional assays we to some extent investigated their pharmacology. First, we observed that PS and 3α5βS did not interact competitively. This conclusion is drawn from the observation that they did not significantly affect one another's modulatory effect curves indicating that PS positive and 3α5βS negative effects on [^{3}H]ifenprodil-specific binding are mediated through distinct sites. The finding obtained when glutamate was coincubated with PS or 3α5βS further supported this. Glutamate decreased PS-induced stimulatory effect by approximately 35% while it had no effect on 3α5βS mediated inhibition. These results show that the binding site for PS is sensitive to the state of the NMDA receptor, an effect that may be mediated through allosteric interaction between the glutamate binding site located on the NR2B subunit (Laube *et al*., 1997) and the

high-affinity PS site. Furthermore, it is consistent with the findings in electrophysiological studies where the positive modulatory effect of PS also is decreased during the activated state of the receptor (Horak *et al*., 2004). When present alone, glutamate increased [3H]ifenprodil binding, an effect that has also been reported by others (Grimwood *et al*., 2000), although in contrast to the inhibitory effect of AP5 as reported in that study, we did not observe any effect of this glutamate site antagonist on neither unmodulated or neurosteroid modulated [3H]ifenprodil binding. Thus, our results further underline previous findings that ifenprodil binds with higher affinity to activated NMDA receptors (state-dependent binding) (Kew *et al*., 1996). The fact that glutamate had no effect on 3α5βS-induced changes of [3H]ifenprodil binding further support that its site is distinct from that of PS and suggests that the action of 3α5βS is not dependent on the state of the NMDA receptor. The effect of NMDA receptor ligands acting at other modulatory sites was also studied. Reduction of two cysteine residues in the NR1 subunit is shown to potentiate the NMDA receptor response (Sullivan *et al*., 1994). Such modification of the receptor complex, induced by the reducing agent DTT, had no effect on the modulation of [3H]ifenprodil binding by PS or 3α5βS, Finally, the effects of the two neurosteroids were found to be unaffected in the presence of the essential coagonist glycine which binding site resides exclusively on the NR1 subunit (Kuryatov *et al*., 1994; Hirai *et al*., 1996). When summing up these characteristics for the sites mediating the effects of the modulators PS and 3α5βS on [3H]ifenprodil binding, it is obvious that the pharmacology show many similarities with that observed in electrophysiological experiments. In such studies on chick spinal cord neurons and NMDA receptor expressing *Xenopus laevis* oocytes, PS and 3α5βS were demonstrated also to act through different sites (Park-Chung *et al*., 1997). Furthermore, the steroids are shown not to interact with the redox modulatory or the glycine site (Wu *et al*., 1991; Park-Chung *et al*., 1994; 1997). The interaction between PS (at the low-affinity site) and glutamate on one hand and ifenprodil and glutamate on the other, have been suggested to be mediated through conformational changes of their binding sites located on the NR2B subunit (Grimwood *et al*., 2000; Horak *et al*., 2004). Since we here observe interactions comparable to those found by these authors and no effect of the NR1-specific ligand glycine, it is tempting to speculate that the high-affinity site for PS, also is located on the NR2B subunit. Furthermore, as none of the allosteric ligands tested affected 3α5βS mediated modulation of [3H]ifenprodil binding, it is likely that this steroid act on a site not allosterically linked to any of those recognised by these ligands.

Receptor-binding techniques can be a powerful tool for the detection of allosteric interactions and have often been used as such in studies on G-protein coupled receptors (Christopoulos & Kenakin, 2002; Jensen & Spalding, 2004). A sensitive detector for allosteric effects is the kinetics for ligand dissociation and any change in the determined rate is believed to mirror receptor allosterism. The decreased dissociation rate for [3H]ifenprodil seen in the presence of PS thus suggests a positive allosteric effect on the affinity for this radioligand. In the case of 3α5βS, the steroid-induced two-site model observed in the saturation and displacement experiment was confirmed by a fast and a slow dissociation constant. The observation that PS and 3α5βS can induce a shift in the binding kinetics from a one-site into a two-site affinity fit further support conformational changes of the ifenprodil binding site. This may also explain the increase in B_{max} for [3H]ifenprodil seen in the presence of PS. Eventhough ifenprodil is suggested to bind to a single high-affinity site on the NR2B subunit (Perin-Dureau *et al*., 2002), the neurosteroids may induce conformational changes exposing new sites for the antagonist or modulate populations of NR1/NR2B channels with sites for [3H]ifenprodil that for some reason were not previously exposed. Notably, one of the first reports on modulation of the GABA receptor showed that the presence of an anaesthetic steroid enhanced [3H]muscimol binding to rat brain membranes, modifying the kinetics from a two-site into a better one-site fit (Harrison & Simmonds, 1984).

PS and 3α5βS display bell- or U-shaped dose–effect curves, respectively, on [3H]ifenprodil binding to the cortical membranes (Figure 1). Interestingly, low doses of PS induced improvement of cognitive functions which were also described by an biphasic dose–response curve (Flood *et al*., 1995). Furthermore, biphasic effects have also been demonstrated to occur for other modulating factors acting on the NMDA receptor. For example, low doses of cations or polyamines stimulate binding of the noncompetitive channel blocker [3H]MK-801 and the NMDA site selective antagonist [3H]CGP 39653 to rat brain membranes while high doses reduce such binding (Mukhin *et al*., 1997). A similar effect has also been described for polyamines in studies on [3H]MK-801 binding to porcine hippocampal membranes (Hofner & Wanner, 2000). Moreover, zinc has been demonstrated to display bell-shaped dose–response curves in desensitisation experiments measuring calcium current in HEK-293 cells expressing NR1/NR2A receptors (Zheng *et al*., 2001). This phenomenon may reflect high- and low-affinity states of the same binding site or distinct sites with different affinities for the modulators.

In conclusion, the present study shows that low amounts of PS or 3α5βS change [3H]ifenprodil binding kinetics in a fashion compatible to an allosteric modulation. Ifenprodil and some derivatives have been found to be neuroprotective, an effect that may be mediated through blockade of the NR1/NR2B form of NMDA receptors (for reviews see Wang & Shuaib, 2005). This family of compounds are attractive therapeutic agents with unique advantages to conventional agonists and antagonists due to their state-dependent action; however, their affinity for several other receptors have limited their clinical use. In this context, the effect of PS to stimulate [3H]ifenprodil binding would be of clinical interest since a maintained effect at decreased doses of the NR2B antagonist may reduce its side effects. Thus, a therapeutic approach combining PS and ifenprodil may lower the dose of the latter compound. Apart from the action on [3H]ifenprodil binding, the functional consequences of this neurosteroid modulation remain to be elucidated since no endogenous compound so far has been found to specifically interact with the binding site for ifenprodil.

This study was founded by the Swedish Medical Research Council (Grant 9459). We thank Dr Qin Zhou for her help in animal management and Dr Staffan Uhlén for his help with receptor-binding theories.

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(Received July 1, 2005
Revised August 16, 2005
Accepted August 18, 2005
Published online 19 September 2005)