

# Distinct Sites for Inverse Modulation of *N*-Methyl-D-Aspartate Receptors by Sulfated Steroids

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## SUMMARY

Steroid sulfation occurs in nervous tissue and endogenous sulfated steroids can act as positive or negative modulators of *N*-methyl-D-aspartate (NMDA) receptor function. In the current study, structure-activity relationships for sulfated steroids were examined in voltage-clamped chick spinal cord and rat hippocampal neurons in culture and in *Xenopus laevis* oocytes expressing NR1<sub>100</sub> and NR2A subunits. The ability of pregnenolone sulfate (a positive modulator) and epipregnanolone sulfate (a negative modulator) to compete with each another, as

well as with other known classes of NMDA receptor modulators, was examined. The results show that steroid positive and negative modulators act at specific, extracellularly directed sites that are distinct from one another and from the spermine, redox, glycine, Mg<sup>2+</sup>, MK-801, and arachidonic acid sites. Sulfated steroids are effective as modulators of ongoing glutamate-mediated synaptic transmission, which is consistent with their possible role as endogenous neuromodulators in the CNS.

Nervous tissue synthesizes numerous steroids from cholesterol. Such neurosteroids have been proposed to control neuronal excitability by modulating ligand and/or voltage-gated ion channels (1, 2). Increasing evidence suggests that certain steroids also play a critical role in important physiological processes such as learning, memory, and aging (3, 4). This hypothesis is consistent with the findings that the levels of the neurosteroids dehydroepiandrosterone and its sulfate, DHEAS, are decreased with age (5) and DHEAS improves memory in aging mice (6). In addition, the risk of Alzheimer's disease and related dementia has been shown to be decreased in women who have received estrogen replacement therapy (7).

PS, one of the most abundant neurosteroids, has diverse modulatory effects on ligand-gated ion channels, acting as a negative modulator of  $\gamma$ -aminobutyric acid<sub>A</sub> (8, 9), glycine (10), kainate, and AMPA receptors, while positively modulating the NMDA receptor (11). PS and some structurally

related derivatives also augment NMDA receptor-mediated increases in intracellular Ca<sup>2+</sup> in cultured rat hippocampal neurons (12, 13). Behavioral studies have shown that PS increases the convulsant potency of NMDA (14) and enhances memory retention in mice (3, 15) and memory performance in the rat when injected directly into the nucleus basalis magnocellularis (16). PS also prevents NMDA receptor antagonist-induced deficits in a passive avoidance memory task (17) and antagonizes dizocilpine-induced amnesia in rats (18). Chronic inhibition of steroid sulfatase activity by estrone sulfamate enhances passive avoidance memory (19). These observations suggest that the behavior of animals after PS administration or after inhibition of sulfatase activity is altered in a manner that is consistent with positive modulation of NMDA receptor function.

In contrast, 3 $\alpha$ 5 $\beta$ S (pregnanolone sulfate) inhibits the NMDA response by a voltage-independent, noncompetitive mechanism (20). However, little is known about the steroid modulatory site or sites of the NMDA receptor. In particular, it is not known whether positive and negative steroid modulators of the NMDA receptor act at the same site, nor is it known whether the site or sites of steroid action correspond to the sites of action of other known NMDA receptor modu-

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**ABBREVIATIONS:** NMDA, *N*-methyl-D-aspartate; DHEAS, dehydroepiandrosterone sulfate; PS, pregnenolone sulfate; 3 $\beta$ 5 $\beta$ S, 3 $\beta$ -hydroxy-5 $\beta$ -pregnan-20-one sulfate; AMPA,  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; EPSC, spontaneous excitatory postsynaptic current; ara-C, 1- $\beta$ -D-arabinofuranosylcytosine; PHS, pregnenolone hemisuccinate; EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; APV, ( $\pm$ )-2-amino-5-phosphonopentanoic acid; 3 $\alpha$ 5 $\beta$ S, 3 $\alpha$ -hydroxy-5 $\beta$ -pregnan-20-one sulfate; 3 $\beta$ 5 $\alpha$ S, 3 $\beta$ -hydroxy-5 $\alpha$ -pregnan-20-one sulfate; 3 $\alpha$ 5 $\alpha$ S, 3 $\alpha$ -hydroxy-5 $\alpha$ -pregnan-20-one sulfate.

lators, such as polyamines (21), redox agents (22), arachidonic acid (23), and MK-801 (24).

In the current study, we examined the effects of sulfated steroids on the current induced by NMDA in primary cultures of voltage-clamped chick spinal cord, rat hippocampal neurons, and NR1<sub>100</sub> + NR2A subunits expressed in *Xenopus laevis* oocytes. A variety of sulfated steroids modulate the NMDA response in either a positive or negative direction with a high degree of structural specificity. 3 $\beta$ 5 $\beta$ S (epipregnanolone sulfate), a PS analog, inhibits the NMDA response. 3 $\beta$ 5 $\beta$ S and PS do not act through the spermine, arachidonic acid, or redox sites of the NMDA receptor, suggesting that these sulfated steroids act through a unique modulatory site or sites associated with the NMDA receptor. Surprisingly, the interaction between PS and 3 $\beta$ 5 $\beta$ S is not competitive, demonstrating the presence of independent pathways for negative as well as positive modulation of the NMDA response. Furthermore, the modulation of spontaneous EPSCs by PS and 3 $\beta$ 5 $\beta$ S demonstrates that these steroids have the capacity to exert powerful effects on excitatory synaptic transmission and brain excitability.

## Experimental Procedures

**Tissue culture.** Cultures of spinal cord neurons were prepared from 7-day chick embryos as described previously (10). Cultures of hippocampal neurons were prepared as described by Brewer (25) with some modifications. Dissociated hippocampal cells from 18-day Sprague-Dawley rat embryos were plated onto 35-mm culture dishes in Dulbecco's modified Eagle's medium supplemented with 2.4 mg/ml bovine serum albumin, 26.5 mM sodium bicarbonate, 1 mM sodium pyruvate, 20 mM HEPES, 10% fetal bovine serum, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and a modification of Brewster B16 defined components (with 250 nM vitamin B12 and without catalase, glutathione, and superoxide dismutase). All cultures were maintained in a humidified atmosphere of 5% CO<sub>2</sub>/95% air at 37°. Non-neuronal cell division was inhibited by exposure to 10<sup>-6</sup> M ara-C. The ara-C was added to spinal cord cultures 36 hr after plating. This medium was removed 1 day later and replaced with fresh medium. Cultured chick spinal cord neurons were used in experiments 2–4 weeks after plating. The ara-C was added to rat hippocampal cultures 48 hr after plating. This medium was removed 48 hr later and replaced with serum-free Dulbecco's modified Eagle's medium plus defined components. Cultured rat hippocampal neurons were used for experiments 14–18 days after plating.

**Whole-cell current recordings.** Whole-cell currents were recorded by the whole-cell variant of the patch-clamp technique (26). Patch electrodes were fabricated with a double pull from borosilicate glass microcapillary pipettes (Fisher Scientific, Fair Lawn, NJ) on a David Kopf vertical pipette puller (model 700D). Electrode resistance was 4.5  $\pm$  0.06 M $\Omega$  (57 microelectrodes) when filled with intracellular solution. The electrode solution usually contained 150 mM CsCl, 3 mM NaCl, 11 mM EGTA, and 10 mM HEPES (pH adjusted to 7.2 with CsOH). To measure spontaneous excitatory synaptic currents, a low Cl<sup>-</sup> concentration (10 mM) pipette solution (10 mM KCl, 3 mM sodium gluconate, 140 mM potassium gluconate, 11 mM EGTA and 10 mM HEPES, pH adjusted to 7.2 with KOH) was used to inhibit Cl<sup>-</sup> currents. The bath solution contained 150 mM NaCl, 4 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 10 mM HEPES, pH adjusted to 7.2 with NaOH. All experiments were performed at room temperature (23–25°) and, except where otherwise noted, at a holding potential of -70 mV.

Recordings were made using an Axopatch 1B patch-clamp amplifier (Axon Instruments, Burlingame, CA). Series resistance, which initially measured 6.3  $\pm$  0.13 M $\Omega$  (57 neurons), was compensated (>53%). Currents were filtered at 1 kHz using an eight-pole Bessel

filter and digitized (40 msec/point) using an on-line data acquisition system (pClamp; Axon Instruments). To measure cell capacitance, a hyperpolarizing step (5 mV, 8 msec) was applied, and the area of the resultant capacitative current spike was calculated by numerical integration.

Pregnenolone hemisuccinate methyl ester was prepared by treatment of the parent compound in methanol at 0° with an ethereal solution of diazomethane in the usual manner. PHS and PS (sodium salt) were obtained from Steraloids (Wilton, NH). All other steroid sulfates were synthesized as their trimethylammonium salts according to the procedure of Dusza *et al.* (27). Their structures were established by elemental analysis and NMR spectroscopy. Three-dimensional structural models were constructed by energy minimization using RASMOL.

Stock solutions of steroids were prepared in dimethylsulfoxide (final concentration, 0.5% v/v). All other drug solutions, including NMDA and external buffer (in the pressure pipette), also contained 0.5% dimethylsulfoxide, which by itself had little or no effect on the NMDA-induced current. All other drugs were obtained from Sigma, with the exception of spermine tetrahydrochloride (Aldrich).

Drug solutions were applied to single neurons by pressure ejection (15 p.s.i.) from seven-barrel pipettes (28, 29). The drug solution in the pressure pipette rapidly and effectively replaces the solution surrounding the target neuron, with <10% dilution. To measure responses to exogenously applied agonists, neurons received a 10-sec prepulse of either external buffer or drug solution, followed by a 10-sec application of agonist or agonist plus drug, followed by a 10–20-sec pulse of external buffer solution. A period of 2–3 min was allowed between successive applications of agonist. For spontaneous excitatory postsynaptic current experiment, drugs were applied to the target neuron by pressure ejection for a total of 80 sec; spontaneous activity from the second half of the application period was analyzed for each neuron. For each experiment, two 40-sec traces were analyzed for each neuron. The target neuron was washed by pressure application of buffer for 2–3 min between drug applications.

**Oocyte electrophysiology: *X. laevis* expression system.** mRNA was prepared through *in vitro* transcription of NR1<sub>100</sub> and NR2A cDNAs using the mMessage mMachine kit (Ambion, TX). NR1<sub>100</sub> and NR2A clones were kindly provided by Dr. R. S. Zukin (Albert Einstein College of Medicine, New York, NY) and Dr. S. Nakanishi (Faculty of Medicine, Kyoto University, Kyoto, Japan), respectively. The oocytes were removed from *X. laevis* frogs. After defolliculation, isolated oocytes were transferred into glass petri dishes containing ND96 solution [96 mM NaCl, 1 mM MgCl<sub>2</sub>, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>, 5 mM HEPES, 2.5 mM pyruvate (pH adjusted to 7.4 with NaOH)] and were maintained in an incubator at 18°. On the following day, batches of 20–40 selected oocytes were injected with 50 nl of prepared RNA solution (0.5 ng of NR1 and 12 ng of NR2A mRNAs/oocyte). The injected oocytes were used for electrophysiological experiments after a 4–10-day incubation at 18°.

**Electrical recordings and drug application.** Recordings from *X. laevis* oocytes were obtained in two electrode voltage-clamp mode using an Axoclamp-2A amplifier (Axon Instruments). The microelectrodes were fabricated from glass capillaries and were filled with 3 M KCl solution. The resistance of filled microelectrodes was 2.5–3.5 M $\Omega$ . The oocytes were clamped at a holding potential of -70 mV. The membrane current was filtered at 500 Hz and sampled at 100 Hz frequency. The drugs were applied using a gravity-driven external perfusion system. The data acquisition and external perfusion control were done using custom-written software implemented in the SuperScopeII programming language (GW Instruments, Somerville, MA).

The degree of modulation of the amino acid response by steroids was expressed as the percent change, defined as (I'/I - 1)  $\times$  100%, where I and I' are, respectively, the agonist-induced currents in the absence and presence of steroid. Throughout, results are expressed as mean  $\pm$  standard error; statistical comparison of groups was carried out using Student's *t* test.

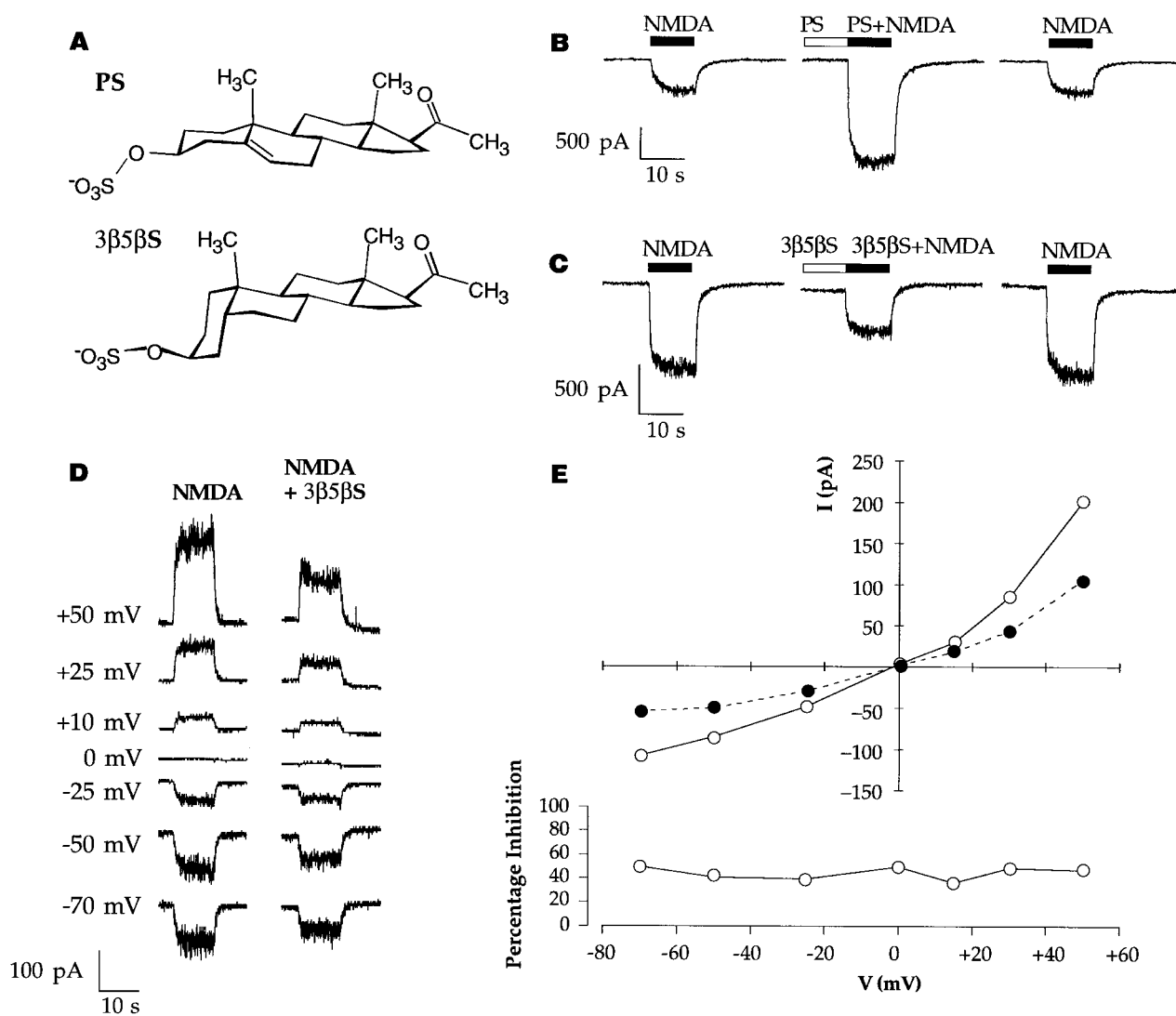
## Results

**Bidirectional modulation of the NMDA receptor by PS and 3 $\beta$ 5 $\beta$ S.** Currents elicited by 30  $\mu$ M NMDA were recorded in primary cultures of chick spinal cord neurons by whole-cell recording. At a holding potential of  $-70$  mV, 100  $\mu$ M PS increases the amplitude of the NMDA-induced whole-cell current by  $\sim 2.5$  fold ( $150 \pm 14\%$  potentiation, 14 neurons; Fig. 1B). In contrast, 3 $\beta$ 5 $\beta$ S, an analog of PS, inhibits ( $50 \pm 4\%$ , 12 neurons) the NMDA response (Fig. 1C). 3 $\alpha$ 5 $\beta$ S (100  $\mu$ M) has a similar inhibitory effect ( $66 \pm 3\%$ , 5 neurons; Park-Chung *et al.*, 1994), whereas 3 $\beta$ 5 $\alpha$ S (100  $\mu$ M) potentiates ( $39 \pm 6\%$ , 7 neurons) the NMDA response, demonstrating that the interaction of 3 $\beta$ 5 $\beta$ S with the NMDA receptor is stereospecific about C5.

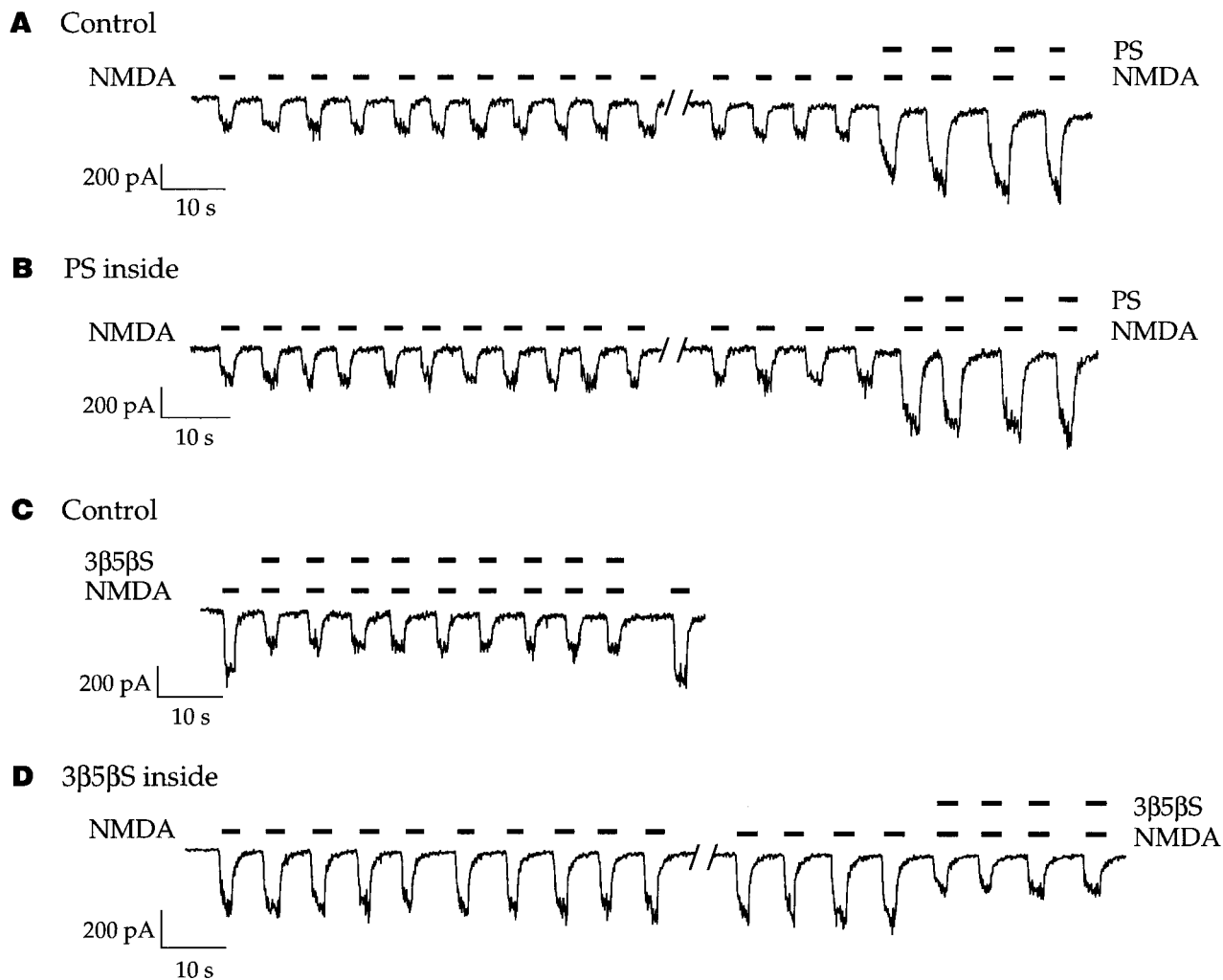
Both onset of inhibition of the NMDA response by extracellular 3 $\beta$ 5 $\beta$ S and recovery from inhibition are rapid, with no evidence of use dependence (Fig. 2C), in contrast to MK-

801 (24), which in a similar experiment exhibits clear use dependence (data not shown). To determine whether inhibition of the NMDA response by 3 $\beta$ 5 $\beta$ S is voltage dependent, we constructed current/voltage curves for the NMDA response in the presence and absence of 3 $\beta$ 5 $\beta$ S. As shown in Fig. 1, D and E, the percentage inhibition of the NMDA response is independent of holding potential, indicating the absence of voltage-dependence.

**PS and 3 $\beta$ 5 $\beta$ S act through extracellularly directed sites.** When PS (100  $\mu$ M) is applied intracellularly by inclusion in the electrode buffer, the average NMDA response does not differ significantly from control and remains stable throughout the recording period (3–5 min; Fig. 2B). The same result is obtained when NMDA responses are normalized to the cell capacitance (pF) to adjust for cell-to-cell variability in the membrane area, demonstrating that intracellular PS cannot gain access to the steroid modulatory site of the



**Fig. 1.** Bidirectional modulation of the NMDA receptor by steroids. A, Structural comparison of PS and 3 $\beta$ 5 $\beta$ S, based on three-dimensional modeling by RASMOL energy minimization. B, PS (100  $\mu$ M) reversibly potentiates the current induced by 30  $\mu$ M NMDA. C, 3 $\beta$ 5 $\beta$ S (100  $\mu$ M) inhibits the current induced by 30  $\mu$ M NMDA. D, The 30  $\mu$ M NMDA-induced current is inhibited by 3 $\beta$ 5 $\beta$ S (100  $\mu$ M) at both positive and negative potentials. E, Current-voltage plot for the NMDA (30  $\mu$ M)-induced current in the presence and absence of 100  $\mu$ M 3 $\beta$ 5 $\beta$ S showing the absence of voltage dependence of inhibition. The holding potential was stepped from  $-70$  mV to the indicated voltage. NMDA was applied after the current stabilized. Results are from the neuron shown in D; similar results were obtained with three other neurons.



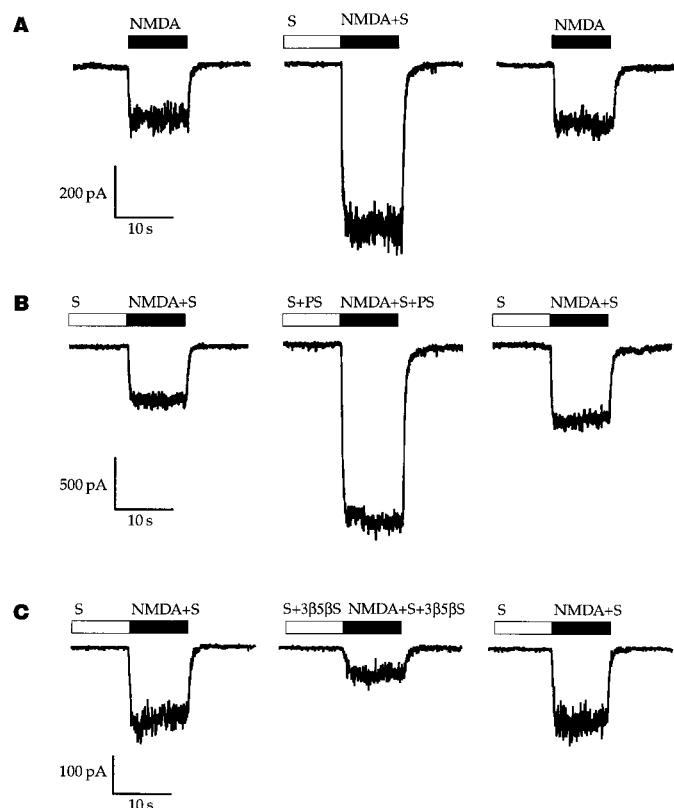
**Fig. 2.** Intracellular steroids do not alter modulation of the NMDA response by steroids applied extracellularly. **A**, Consecutive records of 30  $\mu\text{M}$  NMDA-induced currents showing potentiation of NMDA responses by 100  $\mu\text{M}$  PS ( $158 \pm 24\%$ , 4 neurons) in the absence of intracellular PS. Every 5 sec, 30  $\mu\text{M}$  NMDA with or without 100  $\mu\text{M}$  PS was applied for 2 sec throughout the recording period (4–5 min). The break (//) represents an additional 80 sec of recording between two traces. **B**, Records of 30  $\mu\text{M}$  NMDA-induced currents and potentiation by 100  $\mu\text{M}$  PS ( $152 \pm 23\%$ , 4 neurons) in the presence of intracellular 100  $\mu\text{M}$  PS. The average NMDA response does not differ significantly from control ( $235 \pm 58$  pA, 12 neurons, with PS inside versus  $267 \pm 31$  pA, 19 neurons, without PS). The same result is obtained when NMDA responses are normalized to the cell capacitance to adjust for cell-to-cell variability in membrane area ( $6.5 \pm 0.9$  pA/pF, 4 neurons, with PS inside versus  $8.6 \pm 1.7$  pA/pF, 6 neurons, without PS). **C**, Use-independent inhibition of 30  $\mu\text{M}$  NMDA-induced currents by extracellularly applied 100  $\mu\text{M}$  3 $\beta$ 5 $\beta$ S ( $48 \pm 3\%$ , 5 neurons). **D**, Records of 30  $\mu\text{M}$  NMDA-induced currents showing inhibition by 100  $\mu\text{M}$  3 $\beta$ 5 $\beta$ S ( $44 \pm 4\%$ , 7 neurons) in the presence of intracellular 200  $\mu\text{M}$  3 $\beta$ 5 $\beta$ S. The average NMDA response does not differ significantly from control ( $264 \pm 56$  pA, 12 neurons, with 3 $\beta$ 5 $\beta$ S inside versus  $267 \pm 31$  pA, 19 neurons, without 3 $\beta$ 5 $\beta$ S). The same result is obtained when NMDA responses are normalized to the cell capacitance ( $9.4 \pm 2.2$  pA/pF, 4 neurons, with 3 $\beta$ 5 $\beta$ S inside versus  $8.6 \pm 1.7$  pA/pF, 6 neurons, without 3 $\beta$ 5 $\beta$ S).

NMDA receptor. In addition, the ability of PS to potentiate the NMDA response is unaltered in the presence of a high intracellular concentration of PS (Fig. 2, A and B), indicating that PS acts at the extracellular surface of the membrane. Similarly, the addition of 3 $\beta$ 5 $\beta$ S (200  $\mu\text{M}$ ) to the intracellular buffer does not inhibit NMDA-induced currents or prevent inhibition of the NMDA response by extracellular 3 $\beta$ 5 $\beta$ S (100  $\mu\text{M}$ , Fig. 2, C and D), indicating that 3 $\beta$ 5 $\beta$ S also acts at a site associated with the extracellular surface of the membrane.

**Bidirectional modulation is not exerted through the polyamine site.** In agreement with previous results (21), spermine (10–250  $\mu\text{M}$ ) potentiates the NMDA-induced current. Potentiation is maximal ( $136 \pm 33\%$ , four neurons) at a spermine concentration of 100  $\mu\text{M}$  (Fig. 3A). When the concentration of spermine is further increased to 250  $\mu\text{M}$ , it is

less effective in potentiating the NMDA response ( $67 \pm 21\%$ , three neurons; not shown). To determine whether PS, 3 $\beta$ 5 $\beta$ S, and spermine act through a common modulatory site on the NMDA receptor, we examined the effect of PS (100  $\mu\text{M}$ ) on the NMDA response in the presence of a maximally potentiating concentration of spermine (100  $\mu\text{M}$ ). In the absence of spermine, PS potentiates the NMDA response by  $150 \pm 14\%$  (14 neurons), whereas in the presence of spermine, PS potentiates the NMDA induced current by  $178 \pm 12\%$  over the response in the presence of spermine alone (six neurons; Fig. 3B).

The observation that potentiation is not diminished in the presence of spermine strongly suggests that PS and spermine potentiate the NMDA response via different sites. Similarly, Fig. 3C shows that 3 $\beta$ 5 $\beta$ S inhibits the NMDA response by

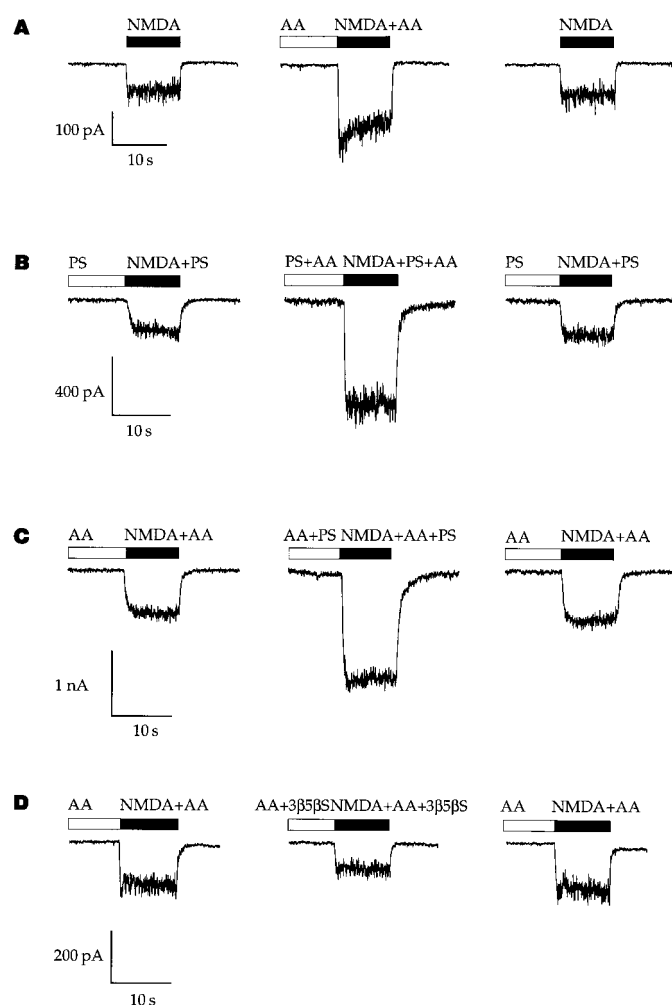


**Fig. 3.** Steroid modulation of the NMDA response is not affected by spermine. **A**, Potentiation of the 30  $\mu\text{M}$  NMDA-induced current by 100  $\mu\text{M}$  spermine (S). **B**, Potentiation of the 30  $\mu\text{M}$  NMDA-induced current by 100  $\mu\text{M}$  PS in the presence of 100  $\mu\text{M}$  spermine. **C**, Inhibition of the NMDA-induced current by 100  $\mu\text{M}$  3 $\beta$ 5 $\beta$ S in the presence of 100  $\mu\text{M}$  spermine.

50  $\pm$  5% (five neurons) in the presence of spermine, which is not significantly different from the percentage inhibition in the absence of spermine. Because this concentration of 3 $\beta$ 5 $\beta$ S is close to its EC<sub>50</sub> (see Fig. 6C), the percentage inhibition by 3 $\beta$ 5 $\beta$ S should be reduced if 3 $\beta$ 5 $\beta$ S and spermine compete for a common site. Therefore, the inhibitory steroid modulatory site is also distinct from the spermine modulatory site.

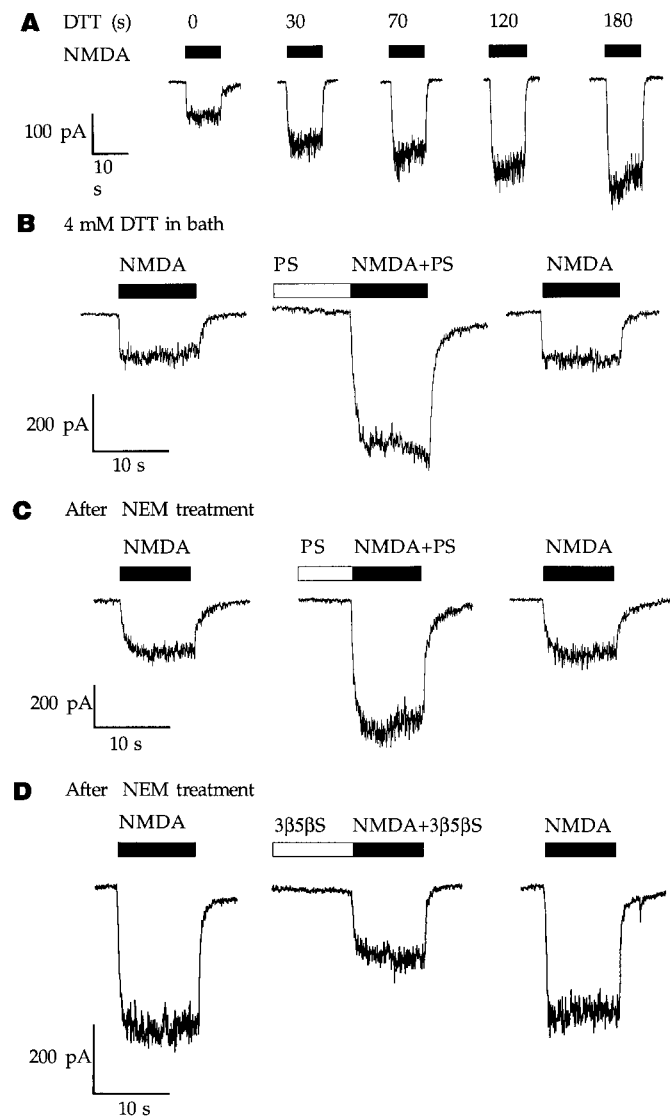
**Bidirectional modulation is not exerted through the arachidonic acid site.** Arachidonic acid potentiates the NMDA response by acting directly at the NMDA receptor (23). Because PS and 3 $\beta$ 5 $\beta$ S have amphiphilic properties, we asked whether these sulfated steroids and arachidonic acid might act through a common site. As shown in Fig. 4A, a maximal concentration of arachidonic acid (10  $\mu\text{M}$ ) potentiates the NMDA response by 120  $\pm$  35% (five neurons) and 158  $\pm$  22% (four neurons) in the absence and presence of 100  $\mu\text{M}$  PS (Fig. 4B). Conversely, PS potentiates the NMDA response by 182  $\pm$  25% (four neurons) in the presence of arachidonic acid (Fig. 4C), which does not differ significantly from potentiation in the absence of arachidonic acid. Similarly, arachidonic acid does not affect the percentage inhibition by 3 $\beta$ 5 $\beta$ S (49  $\pm$  5%, five neurons; Fig. 4D). Thus, these observations demonstrate that the potentiating and inhibitory steroid sites are also distinct from the arachidonic acid modulatory site.

**Bidirectional modulation is not exerted through the redox site.** There is a gradual “run-up” of the NMDA response in the presence of 4 mM DTT, producing a 173  $\pm$  19% (six neurons) enhancement of the NMDA current after 180



**Fig. 4.** Steroid modulation of the NMDA response is not affected by arachidonic acid. **A**, Potentiation of the 30  $\mu\text{M}$  NMDA-induced current by 10  $\mu\text{M}$  arachidonic acid (AA). **B**, Potentiation of the NMDA-induced current by 10  $\mu\text{M}$  arachidonic acid in the presence of 100  $\mu\text{M}$  PS. **C**, Potentiation of the NMDA-induced current by 100  $\mu\text{M}$  PS in the presence of 10  $\mu\text{M}$  arachidonic acid. **D**, Inhibition of the 30  $\mu\text{M}$  NMDA induced-current by 100  $\mu\text{M}$  3 $\beta$ 5 $\beta$ S in the presence of 10  $\mu\text{M}$  arachidonic acid.

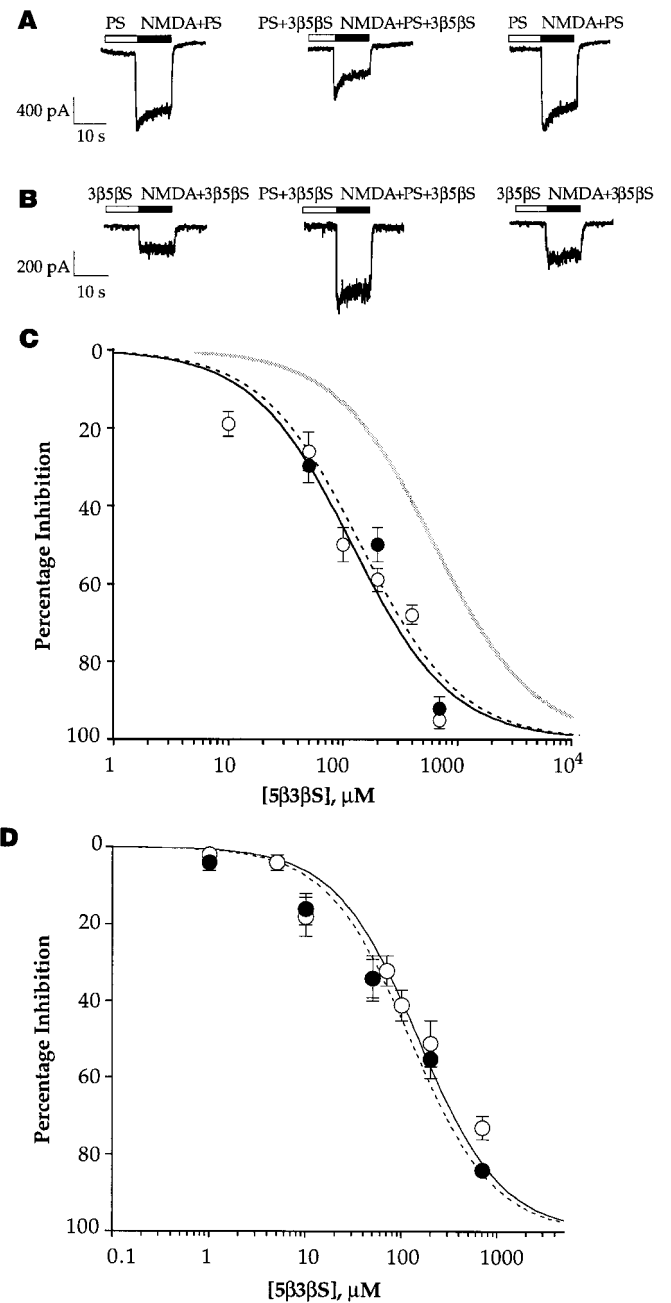
sec of DTT exposure (Fig. 5A). To examine whether PS and/or 3 $\beta$ 5 $\beta$ S interacts with the redox modulatory site of the NMDA receptor, potentiation of the NMDA response by PS was measured after prolonged exposure (1 hr) to DTT (4 mM). Under these conditions, the NMDA response is increased by 169% (326  $\pm$  82 pA, 13 neurons with DTT versus 121  $\pm$  16 pA, 25 neurons without DTT), but PS potentiation of the NMDA response remains unchanged (165  $\pm$  26%, four neurons; Fig. 5B). Similarly, PS potentiation of the NMDA response is not significantly changed (161  $\pm$  12%, four neurons) after exposure to 10 mM DTT for 1 hr (not shown). As an additional test, we treated cells with the sulfhydryl alkylating agent NEM. Cultures were treated for 5 min with 4 mM DTT, followed by 2-min exposure to 4 mM DTT plus 300  $\mu\text{M}$  NEM. Cultures were then washed four times, and potentiation of the 30  $\mu\text{M}$  NMDA response by PS or inhibition by 3 $\beta$ 5 $\beta$ S was measured. Alkylation of thiols with NEM increases the NMDA response (367  $\pm$  82 pA, 25 neurons), compared with control cultures (121  $\pm$  16 pA, 25 neurons),



**Fig. 5.** Steroid modulation of the NMDA response is not affected by redox agents. **A**, Time-dependent increase of the 30  $\mu\text{M}$  NMDA-induced current in the continuous presence of 4 mM DTT. **B**, Potentiation of the 30  $\mu\text{M}$  NMDA-induced current by 100  $\mu\text{M}$  PS after  $\sim 1$  hr of exposure to 4 mM DTT (in the bath). **C**, Potentiation of the 30  $\mu\text{M}$  NMDA-induced current by 100  $\mu\text{M}$  PS after alkylation by treatment with 4 mM DTT for 5 min followed by 4 mM DTT plus 300  $\mu\text{M}$  NEM for 2 min. **D**, Inhibition of the 30  $\mu\text{M}$  NMDA-induced current by 100  $\mu\text{M}$  3 $\beta$ 5 $\beta$ S after alkylation with 4 mM DTT plus 300  $\mu\text{M}$  NEM.

but PS potentiation or 3 $\beta$ 5 $\beta$ S inhibition of the NMDA response remains unchanged (Fig. 5, C and D).

**PS and 3 $\beta$ 5 $\beta$ S act through distinct sites.** To investigate whether the structurally similar steroids 3 $\beta$ 5 $\beta$ S and PS act through the same site to modulate the NMDA response, we examined the effects of 3 $\beta$ 5 $\beta$ S and PS in combination. In the presence of PS, 3 $\beta$ 5 $\beta$ S inhibits the peak NMDA response by  $50 \pm 4\%$  (four neurons, Fig. 6A), which is not significantly different from the inhibition of  $59 \pm 3\%$  (four neurons) measured in the absence of PS. Conversely, potentiation of the NMDA response by PS (100  $\mu\text{M}$ ) is still evident in the presence of 3 $\beta$ 5 $\beta$ S (Fig. 6B). Moreover, the  $\text{EC}_{50}$  for inhibition of the NMDA response by 3 $\beta$ 5 $\beta$ S is similar in the presence and absence of PS (Fig. 6C). Taking the PS  $\text{EC}_{50}$  of 57  $\mu\text{M}$  for potentiation of the NMDA response (11) as an approximation



**Fig. 6.** PS and 3 $\beta$ 5 $\beta$ S act at separate sites on native and recombinant NMDA receptors. **A–C**, Data from primary chick spinal cord neurons. **A**, Inhibition of the NMDA response by 200  $\mu\text{M}$  3 $\beta$ 5 $\beta$ S is not blocked by 200  $\mu\text{M}$  PS. **B**, Potentiation of the NMDA response by 100  $\mu\text{M}$  PS is not blocked by 200  $\mu\text{M}$  3 $\beta$ 5 $\beta$ S. **C**, Concentration-response curve for inhibition of the NMDA-induced current by 3 $\beta$ 5 $\beta$ S in primary chick spinal cord neurons in the absence ( $\circ$ ) and presence ( $\bullet$ ) of 200  $\mu\text{M}$  PS. Results are mean values of pooled data from four to seven neurons. *Error bars*, standard errors. 3 $\beta$ 5 $\beta$ S  $\text{IC}_{50}$  values, estimated by nonlinear regression using the Michaelis-Menten equation, are 120  $\mu\text{M}$  in the absence and 143  $\mu\text{M}$  in the presence of 100  $\mu\text{M}$  PS. *Gray line*, shift in 3 $\beta$ 5 $\beta$ S concentration-response curve expected if interaction between 3 $\beta$ 5 $\beta$ S and PS is competitive. **D**, Concentration-response curve for inhibition of the NMDA-induced current (peak responses) by 3 $\beta$ 5 $\beta$ S in the absence ( $\circ$ ) and presence ( $\bullet$ ) of 200  $\mu\text{M}$  PS in oocytes expressing recombinant NR1<sub>100</sub>/NR2A NMDA receptors. Results are mean values of pooled data from five to eight oocytes. *Error bars*, standard errors. 3 $\beta$ 5 $\beta$ S  $\text{IC}_{50}$  values, estimated by nonlinear regression using the Michaelis-Menten equation, are 151  $\mu\text{M}$  in the absence and 124  $\mu\text{M}$  in the presence of 100  $\mu\text{M}$  PS.

of its  $K_d$ , competitive inhibition would predict that the  $IC_{50}$  for  $3\beta 5\beta S$  measured in the presence of PS would be increased by a factor of 4.5 (Fig. 6C, *gray curve*). These results indicate that  $3\beta 5\beta S$  and PS modulate the NMDA response through distinct sites. In contrast, the enhancing effects of PHS and PS are not additive. PS (200  $\mu M$ ) reduces the ability of PHS (100  $\mu M$ ) to increase the NMDA response (PHS,  $204 \pm 18\%$ , four neurons versus PHS + PS,  $14 \pm 6\%$ , five neurons, not shown).

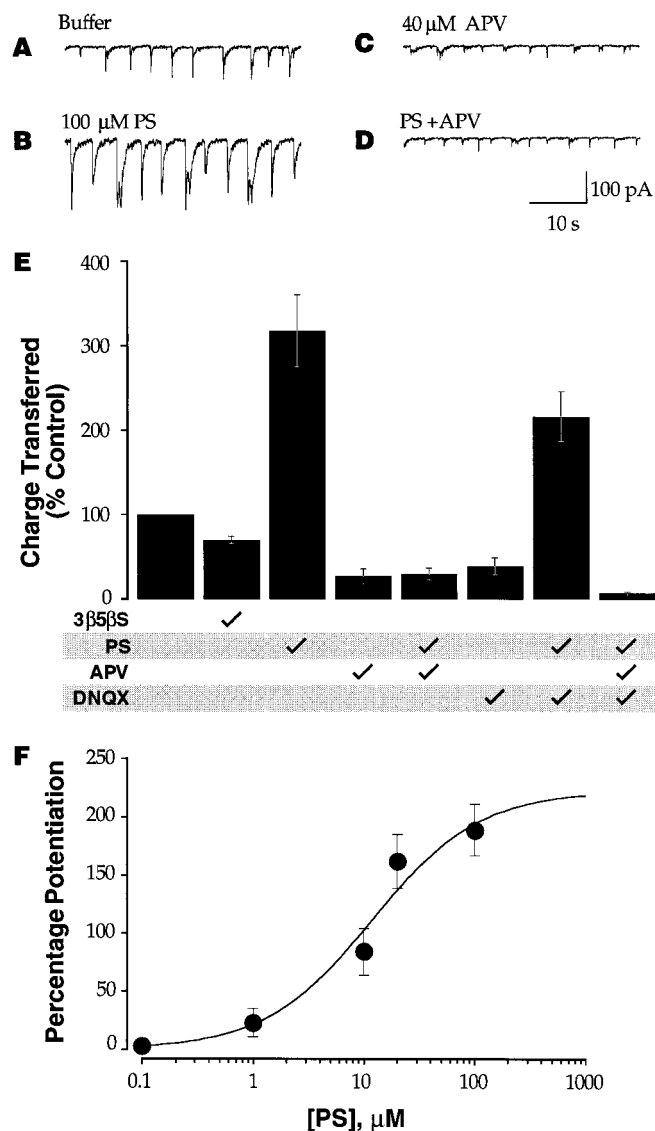
**PS and  $3\beta 5\beta S$  act through distinct sites on NR1<sub>100</sub>/NR2A recombinant NMDA receptors expressed in *X. laevis* oocytes.** To investigate the possibility that PS and  $3\beta 5\beta S$  might be acting on separate receptor populations differing in subunit composition, the modulatory effects of steroids were assessed using oocytes expressing NR1<sub>100</sub> and NR2A subunits. Control oocytes failed to respond to NMDA, whereas oocytes injected with NR1<sub>100</sub> and NR2A subunit mRNA exhibited robust responses to NMDA (30), with an  $EC_{50}$  value of  $62 \pm 4 \mu M$  and a Hill coefficient of  $1.5 \pm 0.1$  (four neurons, not shown). Consistent with reports that potentiation by polyamines is absent with NR1 splice variants bearing the amino-terminal insert (which is present in NR1<sub>100</sub>) or when the NR2A subunit is present (31, 32), NMDA responses of oocytes expressing NR1<sub>100</sub> and NR2A subunits are not potentiated by spermine (0.5–50  $\mu M$ ), whereas higher concentrations of spermine are inhibitory ( $17 \pm 2\%$  inhibition at 100  $\mu M$  spermine). In contrast, oocyte NMDA responses are robustly potentiated by PS ( $257 \pm 44\%$ , five neurons at 100  $\mu M$  PS), providing additional evidence that PS potentiation is not mediated by the polyamine site.

$3\beta 5\beta S$  decreases the maximal NMDA response in oocytes but produces only a slight shift in the NMDA  $EC_{50}$  ( $48 \pm 4 \mu M$ , four neurons; not shown), suggesting that  $3\beta 5\beta S$  inhibits the NMDA response noncompetitively.  $3\beta 5\beta S$  inhibits the peak NMDA response by an equal amount whether in the presence ( $54 \pm 5\%$ ; five neurons) or absence of PS ( $46 \pm 8\%$ ; four neurons). Moreover, there is no significant change in the  $EC_{50}$  for  $3\beta 5\beta S$  inhibition in the presence or absence of 200  $\mu M$  PS (Fig. 6D). These results are consistent with the view that  $3\beta 5\beta S$  inhibits the NMDA response via a site distinct from the PS modulatory site.

**PS or  $3\beta 5\beta S$  potentiates or inhibits spontaneously occurring EPSCs in cultures of rat hippocampal formation.** Next, we considered whether steroids could modulate ongoing synaptic transmission, where exposure to the endogenous neurotransmitter is brief and localized to synaptic receptors. To approach this question, we examined the effects of PS and  $3\beta 5\beta S$  on spontaneously occurring EPSCs in primary cultures of rat hippocampal formation. In the absence of added  $Mg^{2+}$ , cultured hippocampal neurons exhibit frequent spontaneous synaptic activity. By measuring this spontaneous activity, we were able to investigate the effects of steroids on glutamate-mediated synaptic responses. To distinguish EPSCs from IPSCs, the membrane potential was clamped at  $-70$  mV, which is very close to the  $Cl^-$  equilibrium potential. Thus, IPSCs are not expected to be observed and spontaneous activity should consist only of EPSCs. Spontaneously occurring EPSCs are blocked completely by coapplication of the NMDA receptor antagonist APV and the non-NMDA receptor antagonist DNQX (10  $\mu M$ ), indicating that the observed spontaneous activity is glutamate receptor mediated (data not shown). Fig. 7 shows that PS enhances spontaneous EPSCs of neurons in culture. The  $EC_{50}$  for potentiation of EPSCs by PS is 11.9  $\mu M$ . PS augments DNQX-

resistant but not APV-resistant EPSCs, which is consistent with the observation that PS positively modulates the NMDA receptor but not AMPA/kainate receptors (11, 33).

On the other hand,  $3\beta 5\beta S$ , a negative modulator of the NMDA, AMPA, and kainate receptors, inhibits EPSCs (Fig. 7E).  $3\alpha 5\beta S$ , another steroid inhibitor of glutamate responses, also inhibits EPSCs (data not shown).



**Fig. 7.** PS potentiates spontaneous EPSCs of hippocampal neurons. A, Representative control whole-cell recording from a hippocampal neuron showing spontaneous EPSCs. B, Representative trace from the same neuron showing the effect of 100  $\mu M$  PS on spontaneous EPSCs. C, Spontaneous activity is greatly inhibited in the presence of the NMDA receptor antagonist APV (40  $\mu M$ ). D, Residual activity in the presence of APV is not potentiated by PS. E, Histogram summarizing the effects of PS on the integrated EPSCs (charge transferred) and the inhibition of EPSCs by  $3\beta 5\beta S$ . Each bar, mean integrated current from four to six neurons. Results are expressed as percentage of the integrated spontaneous activity recorded from the same neuron during pressure application of buffer. Differences from control are significant for all treatments ( $p < 0.05$ ). Error bars, standard errors. F, Concentration-response curve for potentiation of spontaneous activity by PS, expressed as percentage potentiation of the integrated spontaneous activity recorded in the absence of PS. Smooth curve, fit of the logistic equation. Fitted parameters are  $EC_{50} = 12 \mu M$ , maximal potentiation = 223%, and  $n = 0.9$ .

**Structure-activity relationships for positive and negative modulation by steroids.** Like PS, PHS strongly potentiates the NMDA response, indicating that the 3-sulfate group is not essential for potentiation (Table 1). In contrast, pregnenolone hemisuccinate methyl ester is without activity, suggesting that a negatively charged group at C3 $\beta$  is essential for positive modulation of the NMDA receptor. 11-Keto PS, which differs from PS by the presence of a ketone group at C11, is virtually without effect, whereas 11 $\beta$ -hydroxy PS, with a hydroxyl group at C11, is weakly inhibitory. 7-Keto PS is also inactive. In contrast, compounds with modifications to the C17 side chain, such as 20 $\beta$ -dihydro PS and 21-acetoxy-PS, still potentiate the NMDA response. 17-Hydroxy-PS, which differs from PS by the presence of a hydroxyl group at C17, has activity similar to that of PS. Thus, addition of a C17 hydroxyl to the PS structure does not change its effect on the NMDA response, whereas addition of a keto group or a hydroxyl group at either C7 or C11 results in loss of potentiating activity.

As noted above, both 5 $\beta$ -reduced derivatives of pregnenolone sulfate (3 $\beta$ 5 $\beta$ S and 3 $\alpha$ 5 $\beta$ S) exhibit strong inhibitory effects, whereas 3 $\beta$ 5 $\alpha$ S potentiates the NMDA response.

To examine structural differences of steroids, we constructed three-dimensional structural models by energy minimization using RASMOL. The presence of a C5-C6 double bond results in significant stereochemical differences between PS and 3 $\beta$ 5 $\beta$ S at the ring A/ring B junction and at the sulfate moiety (Fig. 1A).

## Discussion

Sulfated steroids can selectively modulate the actions of NMDA receptors by enhancing or inhibiting NMDA-induced conductance increases (11, 20), but the mechanism by which modulation occurs remains unknown. Previous reports have suggested that an intracellularly directed site may modulate PS action (33, 34), and it is not known whether positive and negative modulatory steroids work at one or more sites on the NMDA receptor. Whether steroids can modulate ongoing synaptic transmission also remains an important question in determining whether such compounds have the potential to act as endogenous neuromodulators. To approach these questions, we used the power of electrophysiology by combining studies of a series of extracellularly and intracellularly applied steroids on vertebrate neurons expressing native NMDA receptors with *X. laevis* oocytes expressing recombinant NR1<sub>100</sub> and NR2A receptor subunits. The results show that there are at least two novel extracellularly directed modulatory sites on the NMDA receptor, one for potentiation and the other for inhibition, that can also enhance or inhibit ongoing synaptic transmission.

**Sulfated steroids modulate the NMDA receptor at a novel site facing the extracellular surface of the membrane.** Previous reports suggest that PS might be able to diffuse within or across the membrane to access its site of action (33, 34). However, we do not observe any significant changes in the average amplitude of the NMDA response when steroids are added intracellularly. Responses to repetitive application of NMDA remain stable for up to 5 min with PS or 3 $\beta$ 5 $\beta$ S present in the intracellular (electrode) buffer, suggesting that intracellular steroids do not modulate the NMDA response even on this extended time scale. The effects

of extracellularly applied PS or 3 $\beta$ 5 $\beta$ S persist when the corresponding steroid is present in the intracellular buffer. These results argue that the sites of action of PS and 3 $\beta$ 5 $\beta$ S are associated with the extracellular surface of the membrane.

The observation that extracellularly applied PS potentiates NMDA responses in an on-cell patch that is not directly exposed to PS (33) might be explained if PS is able to reach its site by lateral diffusion within the plane of the membrane or by a signal transduction mechanism that occurs within the membrane. The results that potentiation of single channel activity occurs in excised inside-out patches after prolonged (1–4 min) PS perfusion of the cytoplasmic patch surface (34) might be explained by a slow leak of PS across the membrane under isolated patch clamp recording conditions. In our experiments, NMDA is applied by extracellular perfusion, so a small amount of PS leaking out through the membrane would not accumulate to a sufficient concentration to produce potentiation.

Because 3 $\beta$ 5 $\beta$ S is charged, voltage-dependence of inhibition would be expected if access to its binding site requires entry into the channel. However, inhibition of the NMDA response by 3 $\beta$ 5 $\beta$ S is not voltage dependent, nor is there any indication of use-dependent inhibition or recovery, such as is seen with MK-801. Considering that 3 $\beta$ 5 $\beta$ S has a greater molecular weight than MK-801 and would be traveling “upstream” in the electric field of the channel, it is hard to imagine that 3 $\beta$ 5 $\beta$ S would move into the channel much faster than MK-801 does. These results argue that inhibition by 3 $\beta$ 5 $\beta$ S is not mediated by the Mg<sup>2+</sup> or MK-801 binding sites.

Potentiation or inhibition of the NMDA response by PS or 3 $\alpha$ 5 $\beta$ S are independent of the glycine modulatory site (11, 20, 33). Similarly, 3 $\beta$ 5 $\beta$ S inhibition of the NMDA response is not reduced in the presence of saturating glycine (data not shown), indicating that its inhibitory effect is not due to competition with endogenous glycine.

A variety of other modulatory sites associated with the NMDA receptor have been proposed, including sites for polyamines, arachidonic acid, and redox agents. Polyamines such as spermine or spermidine at micromolar concentrations increase the NMDA response (21). Arachidonic acid has amphiphilic properties similar to PS and has been proposed to act at the putative fatty acid binding domain of the NMDA receptor (35). In addition, reducing agents enhance NMDA-induced currents, whereas oxidation has the opposite effect (22). Both potentiation of the NMDA response by PS and inhibition by 3 $\beta$ 5 $\beta$ S persist in the presence of high concentrations of spermine or arachidonic acid. Additional evidence against the involvement of the polyamine site is provided by the observation that PS potentiates NMDA responses of *X. laevis* oocytes expressing NR1<sub>100</sub> and NR2A subunits, whereas spermine does not. Taken together, these results argue that the modulatory effects of these steroids are not mediated by either the polyamine or arachidonic acid sites.

Similarly, potentiation by PS and inhibition by 3 $\beta$ 5 $\beta$ S persist following prolonged incubation with DTT or after alkylation with NEM, indicating that excitatory and inhibitory steroids also do not interact with redox modulatory sites. Taken together, these results provide strong support for the existence of a novel extracellularly directed steroid modulatory site(s) on or associated with the NMDA receptor.



TABLE 1

**Effects of steroids on the NMDA response**

Holding potential was  $-70$  mV. NMDA concentration was  $30 \mu\text{M}$ . Concentrations of all steroids were  $100 \mu\text{M}$ . Modulation of the NMDA response is expressed as the percentage of change of the NMDA response in the presence of steroid, as compared to control responses obtained from the same cell before application and after washout of steroid. Values are means  $\pm$  standard errors. The number of cells tested is indicated in parentheses.

| Steroid                                    | Change in response | Steroid   | Change in response |
|--|--------------------|---|--------------------|
| Steroids that potentiate the NMDA response |                    | Steroids that inhibit the NMDA response                     |                    |
|  | %                  |   | %                  |
| PHS  | +204 $\pm$ 18 (4)  | 3 $\alpha$ 5 $\alpha$ S                                     | -25 $\pm$ 3 (8)    |
| 17-Hydroxy-PS                              | +178 $\pm$ 15 (4)  | 3 $\alpha$ -Hydroxy-5 $\beta$ -pregnan-20-one hemisuccinate | -31 $\pm$ 9 (6)    |
| PS   | +150 $\pm$ 14 (14) | 11 $\beta$ -Hydroxy-PS                                      | -31 $\pm$ 2 (3)    |
| 21-Acetoxy-PS                              | +85 $\pm$ 16 (7)   | 3 $\beta$ 5 $\beta$ S                                       | -50 $\pm$ 4 (12)   |
| 20 $\beta$ -Dihydro-PS                     | +92 $\pm$ 17 (5)   | 3 $\alpha$ 5BS  | -66 $\pm$ 3 (5)    |
| 3 $\beta$ 5 $\alpha$ S                     | +39 $\pm$ 6 (7)    | Steroids with minimal activity                              |                    |
|  |                    | 7-Keto-PS   | -5 $\pm$ 5 (4)     |
|  |                    | 11-Keto-PS  | +4 $\pm$ 2 (7)     |
|  |                    | Pregnenolone hemisuccinate methyl ester                     | -0.5 $\pm$ 1.9 (4) |

**Distinct sites for positive and negative modulation of the NMDA receptor by sulfated steroids.** Although PS and  $3\beta5\beta\text{S}$  are structurally similar, they do not interact competitively, arguing that their respective positive and negative modulatory effects are exerted through different sites on or associated with NMDA receptors. This conclusion is based on the following observations: (i) the percentage inhibition of the NMDA response by  $3\beta5\beta\text{S}$  does not change with the addition of a near-maximal concentration of PS; (ii) potentiation by PS of the NMDA response is still evident in the presence of a high concentration of  $3\beta5\beta\text{S}$ ; (iii) there is no significant change in the  $\text{IC}_{50}$  for  $3\beta5\beta\text{S}$  in the presence of PS; and (i.v.) the results cannot be explained by actions of PS and  $3\beta5\beta\text{S}$  on subpopulations of NMDA receptors of different subunit composition, as bidirectional modulation of the NMDA response by PS and  $3\beta5\beta\text{S}$  is also observed in oocytes expressing only NR1<sub>100</sub> and NR2A subunits, and as with neuronal NMDA receptors, the interaction between these two modulators is not competitive.

It remains possible that the NR1<sub>100</sub> and NR2A subunits could assemble into subpopulations containing the same subunits but differing with respect to subunit stoichiometry or arrangement; however, inhibition of the NMDA response at high concentrations of  $3\beta5\beta\text{S}$  is nearly complete, arguing against the presence of a subpopulation of NMDA receptors resistant to  $3\beta5\beta\text{S}$  but sensitive to PS. Moreover, it is possible to achieve nearly complete inhibition of the NMDA response at high concentrations of  $3\beta5\beta\text{S}$ , arguing against the existence of a population of receptors resistant to  $3\beta5\beta\text{S}$  but sensitive to PS.

Thus, there must be at least two distinct steroid modulatory sites with the capacity to modulate NMDA receptor function. Although PS and  $3\beta5\beta\text{S}$  are structurally similar, energy minimization reveals significant differences in their three-dimensional structures (Fig. 1A). This finding further supports our conclusion that PS and  $3\beta5\beta\text{S}$  act at two different sites.

In contrast, potentiation of the NMDA response by PHS is reduced in the presence of PS, suggesting that PS and PHS act at a common site. Because 11-keto PS is structurally similar to PS but has little effect on the NMDA response (Table 1), we tested whether 11-keto PS could act as a steroid site antagonist. In combination studies, 11-keto PS did not antagonize potentiation by PS or inhibition by  $3\beta5\beta\text{S}$  (data not shown), indicating that its inactivity reflects a lack of affinity for the steroid modulatory sites.

**Modulation of EPSCs by steroids.** As it does with chick spinal cord neurons (11), PS enhances by up to 200% the NMDA-induced current of neurons dissociated from embryonic rat hippocampal formation and grown in culture (33). However, the effects of neurosteroids on excitatory synaptic transmission have not been examined previously. Our results demonstrate that PS potentiates spontaneous EPSCs in rat hippocampal neurons, indicating that PS is able to enhance the response of postsynaptic receptors to synaptically released glutamate. No potentiation is observed in the presence of the NMDA antagonist APV, confirming that the potentiation of EPSCs by PS is mediated by NMDA receptors. The  $\text{EC}_{50}$  value for PS modulation of spontaneously occurring EPSCs is about 12  $\mu\text{M}$ , which is lower than the  $\text{EC}_{50}$  of 57.4  $\mu\text{M}$  for PS modulation of the NMDA response in chick spinal cord neurons (11), suggesting that synaptic NMDA receptors

of rat hippocampal neurons may have greater sensitivity to modulation by sulfated steroids.  $3\beta5\beta\text{S}$  and  $3\alpha5\beta\text{S}$  inhibited EPSCs, consistent with their inhibitory effect on the NMDA receptor. It seems unlikely that such bidirectional effects are mediated through a presynaptic mechanism of action. The results strongly suggest that sulfated steroids such as PS and  $3\beta5\beta\text{S}$  have direct neuromodulatory effects on excitatory synaptic transmission in CNS cultures by enhancing or inhibiting postsynaptic NMDA type glutamate receptors.

**Structure-activity relationships for steroid modulators of the NMDA response.** The sulfate group of PS is not essential for potentiation of the NMDA response, but a negatively charged group at C3 is required as potentiating activity is retained in the hemisuccinate but not in the hemisuccinate methyl ester. The addition of a ketone group at C7 or C11 results in complete loss of activity, suggesting that structural requirements for potentiation of the NMDA response are stringent. Of the four possible reduced derivatives of PS,  $3\alpha5\beta\text{S}$ ,  $3\beta5\beta\text{S}$ , and  $3\alpha5\alpha\text{S}$  are inhibitory, whereas  $3\beta5\alpha\text{S}$  potentiates the NMDA response. The opposite effects of the stereoisomers  $3\beta5\alpha\text{S}$  and  $3\beta5\beta\text{S}$  demonstrates that the interaction of these steroids with the NMDA receptor is stereospecific about C5. This stereoselectivity of action argues that  $3\beta5\beta\text{S}$  does not inhibit the NMDA receptor through a nonspecific mechanism, such as perturbation of the lipid bilayer, as proposed for short-chain alcohols (36), but rather acts through a specific modulatory site.

**Physiological and pharmacological significance.** It remains to be determined whether certain NMDA receptor subtypes or spliced variants exhibit a higher affinity for sulfated steroids or whether local concentrations are adequate for modulation to occur under normal conditions.

Because the concentrations of neurosteroids rise in several physiological circumstances such as sexual activity, stress, and during the estrous cycle (1, 37), it is conceivable that PS or one of its sulfated metabolites may contribute to some of the physiological and behavioral changes known to occur in these conditions. Sulfated progesterone metabolites such as  $3\alpha5\alpha\text{S}$ ,  $3\alpha5\beta\text{S}$ , and  $3\beta5\alpha\text{S}$  are present at concentrations as high as 2  $\mu\text{M}$  in the peripheral circulation (38) and the concentration of DHEAS is 8.9  $\mu\text{M}$  in the blood of 40-year-old men (5), but it remains unknown whether sulfated steroids play a role in the functioning of the intact nervous system.

Behavioral studies demonstrate that PS has memory-enhancing effects in mice and rats (3, 16) and prevents NMDA receptor antagonist-induced amnesia (17, 18) and that inhibition of pregnenolone sulfatase activity enhances learning in rats (19). These findings raise the prospect that PS or related sulfated neurosteroids may be active physiologically and useful as a cognitive enhancers.

The inhibitory steroid sulfates such as  $3\beta5\beta\text{S}$ ,  $3\alpha5\beta\text{S}$ , or related compounds may be useful as neuroprotective agents. Release of glycine (39) and arachidonic acid (40) is increased in ischemia, and probably contributes to NMDA receptor-mediated excitotoxicity. Because  $3\beta5\beta\text{S}$  can inhibit the NMDA response in the presence of glycine, polyamines or arachidonic acid, inhibitory steroid sulfates may effectively inhibit excessive NMDA receptor activation during pathophysiological conditions such as stroke.

Our results suggest that the sulfated steroids PS and  $3\beta5\beta\text{S}$  act at unique sites to modulate the NMDA response and that they exert their positive and negative modulation

through independent pathways. These results support the view that these sulfated steroids constitute a novel class of endogenous functional modulators of the NMDA receptor. It will be important to determine whether these steroids can be released from nervous tissue in amounts sufficient to modulate excitatory synaptic transmission and to develop pharmacological antagonists of the steroid modulatory sites to test the hypothesis that sulfated steroids act as physiological neuromodulators *in vivo*. Further characterization of molecular interactions between steroids at specific sites on the NMDA receptor will be of extreme interest due to the critical role of this receptor in many physiological and pathological processes in the central nervous system. Moreover, the development of ligands that act specifically at one or both of these sites should provide a useful strategy for the design of therapeutic agents with neuroprotective, sedative-hypnotic, analgesic, or anesthetic properties.

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