Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS). However, excessive exposure to glutamate and its analogs can result in neuronal death through a process termed excitotoxicity (Olney, 1986). Although the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate-type ionotropic glutamate receptors mediate excitotoxicity under certain conditions (Koh et al., 1990), the N-methyl-d-aspartate (NMDA) receptor has been the major focus of attention. The nature of the negatively charged group attached to the steroid C3 position is important for both the neuroprotection afforded by pregnane steroids and the exacerbation of NMDA-induced neuronal death by pregn-5-enes. Dicarboxylic acid hemiesters of various lengths can substitute for the sulfate group of the positive modulator pregnenolone sulfate and the negative modulator pregnanolone sulfate. This result suggests that precise coordination with the oxygen atoms of the sulfate group is not critical for modulation and that the steroid recognition sites can accommodate bulky substituents at C3. The capacity of charged steroids to enhance or protect against NMDA-induced death of hippocampal neurons is strongly correlated with modulation of NMDA-induced Ca\(^{2+}\) accumulation, indicating that direct enhancement or inhibition of NMDA receptor function is responsible for the proexcitotoxic or neuroprotective effects of these steroids.
Various steroids, including 17β-estradiol (Behl et al., 1995; Miura et al., 1996) and certain synthetic 21-aminosteroids (Monyer et al., 1990), have been shown to have neuroprotective properties, and are thought to antagonize excitotoxicity by scavenging free radicals. However, steroids also may modulate neurotransmitter receptors directly. Pregnenolone sulfate (PS), an abundant neurosteroid (Corpéchot et al., 1983), potentiates NMDA-induced currents (Wu et al., 1991; Bowlbry, 1993), whereas pregnanolone sulfate (3α-hydroxy-5β-pregn-20-one sulfate; 3αβ5S) and 3β-hydroxy-5α-pregnan-20-one sulfate (3β5βS) inhibit NMDA-induced currents (Park-Chung et al., 1994; Yaghoubi et al., 1998). Moreover, the steroid negative modulators 17β-estradiol (Weaver et al., 1997b) and pregnanolone hemisuccinate (3αβSHS; Weaver et al., 1997a) are neuroprotective, whereas the steroid positive modulator PS enhances excitotoxicity (Weaver et al., 1998).

We have shown previously that sulfated steroid positive and negative modulators of the NMDA receptor act through distinct sites to modulate ligand-gated ion channel activity (Park-Chung et al., 1997). In this study, we examine how two different types of structural modifications affect the ability of steroids to interact with these sites to modulate NMDA-induced Ca2+ accumulation and cell death in cultures of hippocampal neurons. Results demonstrate that there is a strong correlation between these two measures of NMDA receptor modulation, and that a more planar versus bent structure is an important determinant of selectivity between the positive and negative modulatory sites associated with the NMDA receptor. In addition, carboxylic acid esters of various lengths in some cases can substitute for sulfate at the C3 position. This is of use in the design of therapeutic agents because the substitution of the hemisuccinate group for sulfate results in a drug that can cross the blood-brain barrier and protect against middle cerebral artery occlusion-induced degeneration of cortical tissue (Weaver et al., 1997a).

**Experimental Procedures**

**Materials.** Steroids were used at 100 μM, except where otherwise stated. PS and 17β-estradiol were obtained commercially from Steraloids (Wilton, NH).

Formate esters were prepared by treating a solution of the steroid (400 mg) in dry dichloromethane (30 ml) with triethylamine (2.4 ml), 4-dimethylpyridine (160 mg), and formic acid (0.32 ml). The mixture was cooled to −20°C and acetic anhydride (1.9 ml) added dropwise over a 30-min period with stirring. The reaction mixture was then warmed to 0°C for 30 min and then the reaction stopped by the addition of methanol (1.0 ml). After evaporation of the solvents in vacuo, the residue was partitioned between ethyl acetate (10 ml) and water (10 ml) and evaporated to dryness. The product was crystallized twice from a mixture of acetone and hexane. The hemialoxamate esters were prepared as described above, but with 568 mg of oxalic acid instead of formic acid. The hemiglutamate esters were prepared as follows: to a solution of steroid (400 mg) in dry pyridine (6 ml), we added glutaric anhydride (400 mg) and 4-pyridinopropionitrile (40 mg). The mixture was allowed to stand at room temperature in the dark for 4 days, when thin-layer chromatography showed complete disappearance of the ester starting material. The reaction mixture was then poured into ice water (20 ml) and the product extracted with ethyl acetate (20 ml), and the extract washed with aqueous 1 N HCl (5 ml) and water (5 ml). After drying the ethyl acetate solution over anhydrous sulfate, the product was treated with activated charcoal (200 mg) and crystallized from a mixture of ethyl acetate and hexane. The hemisuccinate esters were prepared as described above, except that succinic anhydride (225 mg) was used in place of glutaric anhydride, and the reaction required 7 days for completion at room temperature.

Steroids were initially dissolved in 100% dimethyl sulfoxide (DMSO), then diluted into assay buffer at a final DMSO concentration of 0.5% and sonicated for 20 min. All other solutions also contained 0.5% DMSO. Except where otherwise noted, the final steroid concentration was 100 μM. Pregnenolone and 3α5β were used at 20 and 50 μM, respectively, because higher concentrations tended to precipitate in the assay buffer.

**Cell Culture.** Predominantly neuronal cultures were prepared from hippocampal tissue of fetal Sprague-Dawley rats on day 18 of embryonic development as described in Brewer and Cotman (1989).

Briefly, hippocampal cells were dissociated by trituration in Ca2+/ Mg2+-free Hanks’ basic salt solution (Life Technologies, Gaithersburg, MD) supplemented with 4.2 mM bicarbonate, 1 mM sodium pyruvate, 20 mM HEKES, and 3 mg/ml BSA. Dissociated cells were then pelleted by centrifugation (500g; 4 min). The resulting pellet was suspended in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) supplemented with 2.4 mg/ml BSA, 26.5 mM sodium bicarbonate, 1 mM sodium pyruvate, 20 mM HEKES, 10% fetal bovine serum (Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies), and a modification of Brewer’s B16 defined components (with 250 mM vitamin B12 and without catalas, glutathione, and superoxide dmutase; Pike et al., 1990). Cells were then plated onto poly(l-lysine)-coated 24-well culture dishes (Nuncion, Naperville, IL) at a density of 15,000 cell/cm2 and maintained in a humidified atmosphere containing 5% CO2, 95% air at 37°C. After 48 hr, non-neuronal cell division was inhibited by a 48-h exposure to 1 μM cytosine arabinoside. Cultures were subsequently maintained in serum-free DMEM plus defined components and were used for experiments 16 to 24 days after plating. Cultures prepared in this manner contained ~80% neurons, as indicated by staining of non-neuronal cells with antibody to glial fibrillary acidic protein and staining of neurons with antibody to neuron-specific enolase.

**Intracellular Calcium Concentration Measurements.**

NMDA-induced increases in [Ca2+]i, were measured with the Ca2+-sensitive fluorescent dye Fluo-3 AM (Molecular Probes, Eugene, OR) and a Cytofluor 2350 (Perceptive Biosystems, Cambridge, MA) fluorescence plate reader, with excitation and emission filters of 485 and 530 nm, respectively. Hippocampal neurons were loaded with dye by incubating cultures with 10 μM Fluo-3 AM and 0.05% (w/v) Pluronic F-127 (Molecular Probes), a nonionic detergent, for 2 h at 37°C. Fluo-3 AM and Phoronic F-127 were dissolved in DMSO (final concentration of 0.5%). Cultures were then washed three times with assay buffer (120 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 15 mM glucose, 25 mM Tris-HCl, and 0.5 mM tetrodotoxin; pH 7.4) to remove excess dye. For the purposes of calibration, other plate wells were rinsed instead with assay buffer in which 1.8 mM MnCl2 replaced 1.8 mM CaCl2 (Fmin buffer). [Ca2+]i was calculated with the equation:

\[ [Ca^{2+}]_i = K_F [F - F_{min}] /[F_{max} - F], \]

where F is the fluorescence measured, Fmin is the fluorescence in the absence of calcium (determined in Fmin buffer after the addition of 10 μM the Ca2+-ionophore A-23187), Fmax is the fluorescence of the Ca2+-saturated dye (determined in assay buffer after the addition of 10 μM A-23187), and K_F = 320 nM (the equilibrium dissociation constant for the binding of Ca2+ to Fluo-3 AM). Fluorescence measurements were made before and 40 s after the addition of NMDA. Steroid or vehicle (0.5% DMSO) was added 10 min before the addition of NMDA. NMDA was dissolved in DMEM; steroids and A-23187 were dissolved in DMSO. DMSO also was added to controls to maintain a constant final DMSO concentration of 0.5%. Data are expressed as the percentage change in the NMDA-induced increase in [Ca2+]i, in the presence of the indicated steroid. None of the steroids tested significantly altered [Ca2+]i, in the absence of NMDA.
NMDA-Induced Cell Death. Primary cultures of rat hippocampal neurons were exposed to NMDA (dissolved in DMEM) for 15 min (acute exposure) or 16 h (chronic exposure). In acute exposure experiments, cultures were treated with steroid, MK-801 (Research Biochemicals International; dissolved in DMEM), or vehicle during and/or after NMDA exposure. Steroids were dissolved in DMSO (0.5% final concentration), and all treatment media contained 0.5% DMSO. In chronic exposure experiments, cultures were additionally treated with steroid, vehicle, SR-95531 (Research Biochemicals International; dissolved in DMEM), or MK-801 (dissolved in DMEM) during NMDA exposure. After exposure, cultures were washed three times with medium from sister cultures (conditioned medium). After the final wash, steroid or vehicle was reintroduced. Drugs were added to cultures in 25 μl of conditioned medium to yield a final volume of 0.25 ml/well. Except where otherwise noted, final steroid concentration was 100 μM.

The ability of neurons to exclude trypan blue was used to quantitate cell viability (Dawson et al., 1991). Twenty-four hours after acute and 16 h after chronic exposure to NMDA, culture medium was replaced by 0.4% trypan blue in 0.1 M PBS (pH 7.4) and placed in a humidified incubator for 10 min. Cultures were then fixed with 4% paraformaldehyde in PBS for 30 min, at which time the fixative was replaced with PBS. The number of stained and unstained neurons were counted in four high-power fields per culture well with an inverted phase contrast microscope under both bright field and phase contrast settings. At this stage in vitro, neurons were easily distinguishable from non-neuronal cells in vitro by the presence of oval, phase-bright somata and by the morphology of their processes, as confirmed by experiments in which neurons and non-neuronal cells were stained with antibodies as described above. Experiments were performed in triplicate, and all assessments were made with the experimenter blind to the treatment of each culture well. Percentage of cell death is expressed as follows: (number of trypan blue stained neurons/total number of neurons) × 100%. The basal level of neuronal death (termed background), measured in controls lacking NMDA and PS, was 0 to 10% and was subtracted from the raw data in each experiment.

Statistical Analysis. The degree of modulation of NMDA-induced Ca\(^{2+}\) influx and cell death is expressed as the percentage of change, defined as \((I/I' - 1) \times 100\%\), where \(I\) and \(I'\) are the NMDA-induced responses in the absence and presence of modulator, respectively. All data are expressed as mean \pm S.E. Statistical significance was evaluated with 95% CL unless otherwise noted.

Results

Effect of Geometry on Modulation of NMDA Neurotoxicity by Sulfated Steroids. We have identified several reduced metabolites of progesterone that modulate currents evoked by NMDA (Farb and Gibbs, 1996). 3α5βS was the first steroid found to inhibit NMDA-induced currents in cultured neurons (Park-Chung et al., 1994). Consistent with this negative modulation of the NMDA response, 3α5βS (100 μM) reduces the 5 μM NMDA-evoked Ca\(^{2+}\) influx by 32 ± 5% (n = 8) (Fig. 1A). Furthermore, 3α5βS protects neurons from the cell death produced by acute (15 min) exposure to NMDA, raising the EC\(_{50}\) for NMDA-induced neuronal death from 28 to 71 μM and lowering the maximal NMDA-induced excitotoxicity from 80 to 63% cell death (Fig. 1B). This effect is dose dependent, with an EC\(_{50}\) of 45 μM and a 97% maximal inhibition of the cell death induced by 30 μM NMDA (Fig. 1C). The neuronal death caused by chronic (16 h) NMDA treatment also is attenuated by 3α5βS, which, under these conditions, reduces the NMDA E\(_{max}\) from 86 to 70% cell death without affecting the NMDA EC\(_{50}\) (Fig. 1D).

In addition to its effects on the NMDA response, 3α5βS inhibits currents elicited by AMPA and kainate (Park-Chung et al., 1994), raising the possibility that the effect of the steroid on NMDA-induced neuronal death might not be specific to the NMDA receptor. 10 μM 6,7-dinitroquinoxaline-2,3-dione [DNQX, a selective non-NMDA glutamate receptor antagonist (Honore et al., 1988)] and 100 μM SR-95531 (a selective γ-aminobutyric acid\(_A\) receptor antagonist; Farrant and Webster, 1989) have no effect on NMDA-induced neuronal death (data not shown), arguing that the γ-aminobutyric acid\(_A\), AMPA, and kainate receptor types do not play a significant role in this process.

Although 3α5βS nearly eliminates the toxic effects of an acute exposure to 30 μM NMDA, its stereoisomer 3α-hydroxy-5α-pregn-20-one sulfate (3α5αS) is only half as effective, producing a 47 ± 12% (n = 4) inhibition of neuronal death (Fig. 2). Strikingly, whereas 3β5βS reduces NMDA-induced currents and neuronal death (86 ± 3% inhibition; n = 6), its 5α-isomer 3β-hydroxy-5α-pregn-20-one sulfate (3β5αS), both potentiate NMDA-induced currents (Park-Chung, 1997) and exacerbates neuronal death by 40 ± 7% (n = 17). This shows that, as with their effects on NMDA-evoked currents, the neuroprotective effects of these sulfated steroids are contingent on the stereochemistry of the A-B ring junction, whereas stereochemistry at C3 appears to be important only for 5α-isomers.

Effect of Charge and Chain Length on Modulatory Actions of 3α5βS Analogs. To elucidate further the structure-activity relationships for modulation of the NMDA receptor by steroids, we synthesized a series of carboxylic acid derivatives of 3α5β (Fig. 3A). The three negatively charged derivatives, 3α5βHS, pregnanolone hemiosxylate (3α5βHO), and pregnanolone hemisuccinate (3α5βHG) are about equally effective, inhibiting the NMDA-induced rise in [Ca\(^{2+}\)]\(_i\) by ~40% in primary hippocampal cultures, whereas the uncharged 3α5β and pregnanolone formate (3α5βF) have no significant effect on NMDA-induced Ca\(^{2+}\)\(_i\) accumulation (Fig. 3B). Similarly, 3α5βHO, 3α5βHS, and 3α5βHG are neuroprotective, reducing neuronal death caused by acute exposure to 30 μM NMDA by 55 ± 6 (n = 10), 54 ± 3 (n = 24), and 38 ± 6% (n = 9), respectively (Fig. 3C), whereas 3α5β and 3α5βF do not exhibit significant neuroprotective activity.

Effect of Charge and Chain Length on Modulatory Effects of PS Analogs. PS is a potent positive modulator of NMDA receptor function (Wu et al., 1991; Boulby, 1993). To evaluate the role of the sulfate group at C3, we examined the effects of pregnenolone, pregnenolone formate (PF), pregnenolone hemiosxylate (PHO), pregnenolone hemisuccinate (PHS), and pregnenolone hemisulfate (PHG; Fig. 4A).

Potentiation of NMDA-induced Ca\(^{2+}\)\(_i\) accumulation by hippocampal neurons in culture increases with chain length from PHO (62 ± 17%; n = 7) to PHS (113 ± 22%; n = 4), and PHG (146 ± 29%; n = 5), whereas pregnenolone and PF are without effect (Fig. 4B). Similarly, the uncharged pregnenolone and PF are ineffective, whereas the negatively charged PHO, PHS, and PHG exacerbate NMDA-induced cell death by 48 ± 12 (n = 6), 80 ± 16 (n = 9), and 54 ± 9% (n = 6), respectively (Fig. 4C). The effects of these steroids on NMDA-induced excitotoxicity are in general agreement with...
their effects on NMDA-induced Ca\textsuperscript{2+} accumulation, although the contribution of chain length is less clear.

**Discussion**

**Modulation of Ca\textsuperscript{2+} Accumulation Correlates with Excitotoxicity.** The excitotoxicity produced by excessive NMDA receptor stimulation has been implicated in the neurodegeneration associated with a number of CNS diseases and insults (Rothman and Olney, 1986; Gómez-Pinilla et al., 1989; Greenamyre, 1991; Greenamyre and O’Brien, 1991; Weaver et al., 1997a). Evidence indicates that neuronal death results from NMDA receptor-mediated activation of a Ca\textsuperscript{2+}-dependent enzymatic cascade involving lipid peroxidation and protein and DNA degradation (Choi, 1992; Chan, 1996). Our present results, demonstrating that modulation by steroids of NMDA-induced Ca\textsuperscript{2+} uptake is highly correlated with modulation of NMDA-induced neuronal death (Fig. 5), support this view, and indicate that this rapid functional assay can be usefully used to identify steroids with neuroprotective activity.

In this study, we demonstrate that 3α5βS also markedly inhibits NMDA-induced changes in [Ca\textsuperscript{2+}]\textsubscript{i} and neuronal death under both acute and chronic exposure conditions, consistent with our previous finding that 3α5βS inhibits NMDA-induced currents in neurons maintained in primary culture (Park-Chung et al., 1994). It is interesting that, in acute treatments, 3α5βS reduces both the NMDA EC\textsubscript{50} and \( \textit{E}_{\text{max}} \) for causing cell death, while only reducing the \( \textit{E}_{\text{max}} \) in chronic treatments. The reason for this difference is unclear,
but it may indicate metabolic conversion of 3α5βS, such as through the action of a steroid sulfatase, during the course of the chronic treatment, or an adaptive change at the NMDA receptor itself.

**Stereochemistry of NMDA Receptor Modulation.** To investigate the structural requirements for steroid inhibition of NMDA-induced neuronal death, 3α5β and stereoisomers of 3α5βS were assayed for activity, as were several related synthetic pregnane steroids. 3β5αS is as effective as 3α5βS at protecting against the neuronal death produced by acute exposure to NMDA. This suggests that the stereochemistry at C3 is not critical for inhibition of NMDA-induced neuronal death by the C5β-pregnane isomers. Notably, the isomer with 5α-stereochemistry, 3α5αS, exhibits reduced neuroprotective activity compared with the 5β-isomer. 3α5αS is about half as effective as 3α5βS and 3β5βS in protecting against NMDA-induced cell death, whereas 3β5αS actually exacerbates the toxicity of NMDA and potentiates the NMDA-induced elevation of [Ca²⁺]. These results are in agreement with previous electrophysiological studies of voltage-clamped chick spinal cord neurons in primary culture (Fig. 2), in which 3α5βS and 3β5βS are strong inhibitors of the NMDA-induced current, 3α5αS is a weaker inhibitor, and 3β5αS weakly potentiates the NMDA response (Park-Chung et al., 1997). The results indicate that stereochemistry at the A-B ring junction is an important determinant of the activity of pregnanes with a negatively charged group at C3. The effect of 5α-stereochemistry on the structure of the steroid molecule is to flatten out the ring system into a more planar configuration, much like the effect of the C5–C6 double bond in the pregn-5-ene series (Fig. 2). Because competition experiments indicate that positive and negative modulation by steroids are mediated by distinct sites (Park-Chung et al., 1997), it seems likely that the more planar ring structure of the pregn-5-enes and 5α-pregnanes improves the fit of the steroid molecule to the potentiating site and/or impairs its fit into the inhibitory site.

**Fig. 2.** Inhibition of NMDA-induced neuronal death by pregnanolone sulfate isomers is stereospecific. The neuronal death caused by acute exposure to 30 μM NMDA is nearly abolished by the two 5β-isomers 3β5βS and 3α5βS. 3α5αS (100 μM) is roughly half as effective, whereas 100 μM 3β5αS potentiates neuronal death. Results are expressed as mean percentage of neuronal death ± S.E., with the number of experiments indicated in parentheses. Previously reported (Wu et al., 1991; Park-Chung et al., 1997; and Park-Chung et al., 1994) modulatory effects on NMDA-induced currents of chick embryonic spinal cord neurons in culture are provided for comparison.
Fig. 3. C3 dicarboxylic acid esters of pregnanolone inhibit NMDA-mediated Ca^{2+} accumulation and neuronal death. A, structures of 3αβ, 3αβS, 3αβF, 3αβHO, 3αβHS, and 3αβHG. Note that 3αβ and 3αβF are uncharged, whereas 3αβS, 3αβF, 3αβHO, 3αβHS, and 3αβHG are negatively charged. B, negatively charged pregnanolone steroids inhibit the 5 μM NMDA-mediated Ca^{2+} influx in rat hippocampal cultures. Columns show mean percentage of reduction of the NMDA-induced rise in [Ca^{2+}]_i in the presence of the indicated steroid (100 μM, except 3αβ, 50 μM). Error bars indicate S.E.M.; the number of experiments is given in parentheses.

Fig. 4. Preg-5-ene steroid-mediated exacerbation of NMDA receptor function is dependent on the C3 ester group. A, structures of pregnenolone (P), PS, PF, PHO, PHS, and PHG. Note that pregnenolone and PF are uncharged, whereas PS, PHO, PHS, and PHG are negatively charged. B, negatively charged pregn-5-ene steroids potentiate the 5 μM NMDA-mediated Ca^{2+} influx in rat hippocampal cultures. Columns show mean percentage of potentiation of the NMDA-induced rise in [Ca^{2+}]_i, in the presence of the indicated steroid (100 μM; except pregnenolone, 20 μM). Error bars indicate S.E.M.; the number of experiments is given in parentheses.

Results are expressed as mean percentage of neuronal death ± S.E. with the number of experiments indicated in parentheses.
charged, is significantly less effective than PHG in enhancing Ca\(^{2+}\) accumulation by hippocampal neurons, suggesting that precise positioning of the negative charge is important for potentiation by pregn-5-ene derivatives. The lower activity of PHO is a bit surprising because PHO has the shortest chain length, such that the position of its charged carboxyl might be expected to be most similar to that of the even smaller sulfate group. However, molecular modeling indicates that the hemisuccinate and hemiglutamate groups have much greater conformational freedom than the hemisuccinate group, perhaps allowing more favorable positioning of the negatively charged carboxyl group in the binding site.

The finding that negatively charged carboxylic acid esters can substitute for the sulfate ester at the C3 position offers prospects for modifying the steroid nucleus to optimize pharmacological and pharmacokinetic properties. Carboxylic acid derivatives of neuroactive steroids may offer improved penetration into the CNS without susceptibility to hydrolysis by sulfatases. This supposition is supported by the observation that 3α5βHS is effective as a hypnotic, anticonvulsant, and anagolic, and reduces the neuronal death that results from middle cerebral artery occlusion in rats, a model of stroke (Weaver et al., 1997a). Thus, it may be possible to rationally design steroidal-based and nonsteroidal therapeutics for the treatment of stroke and disorders arising from the overactivation of the NMDA receptor. Carboxylic acid derivatives of the pregn-5-ene steroids have not been tested in vivo, but based on the present results might be expected to exhibit memory-enhancing effects such as have been described for pregnenolone sulfate (Isaacson et al., 1994; Flood et al., 1995; Vallee et al., 1997).


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