Geometry and Charge Determine Pharmacological Effects of Steroids on N-Methyl-D-aspartate Receptor-Induced Ca$^{2+}$ Accumulation and Cell Death$^1$

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ABSTRACT

Modulation of N-methyl-D-aspartate (NMDA) receptor function by a series of sulfated steroids and dicarboxylic acid ester analogs of pregnenolone sulfate and pregnanolone sulfate was investigated in cultured hippocampal neurons. The "bent" steroid ring structure associated with 5β-stereochemistry favors receptor inhibition, whereas the more planar ring structure of the pregn-5-enes and 5α-pregnanes favors potentiation of NMDA-induced [Ca$^{2+}$] increases and neuronal cell death. 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 pregnanolone sulfate and the negative modulator pregnenolone 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.

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-isoxazolopropionic 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. Under normal physiological conditions, the NMDA receptor plays a vital role in the synaptic plasticity thought to underlie learning and memory (Collingridge and Bliss, 1987), most likely as a result of its ability to transport Ca$^{2+}$ (Mac Dermott et al., 1986), which acts as an intracellular second messenger. However, overstimulation of NMDA receptors can disrupt Ca$^{2+}$ homeostasis, resulting in an elevated [Ca$^{2+}$], which can trigger a number of deleterious Ca$^{2+}$-dependent enzymatic cascades, including activation of proteases (Siman and Nozek, 1988; Rami et al., 1997) and endonucleases (Joseph et al., 1993) and generation of oxygen free radicals (Beckman et al., 1990; Dawson et al., 1991; Heinzel et al., 1992), ultimately resulting in cell death.

NMDA receptor-mediated neuronal death has been linked to certain neurodegenerative diseases (Choi, 1988; Young et al., 1988; Greenamyre and Young, 1989; Greenamyre and O’Brien, 1991) as well as to the neurodegeneration associated with hypoxic-ischemic events (Rothman and Olney, 1986) and trauma (Gómez-Pinilla et al., 1989). Selective NMDA receptor antagonists are able to inhibit the Ca$^{2+}$-dependent neuronal death caused by hypoxia-ischemia and glutamate exposure (Choi et al., 1988).

ABBREVIATIONS: CNS, central nervous system; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid; NMDA, N-methyl-D-aspartate; PS, pregnenolone sulfate; 3αβS, 3α-hydroxy-5β-pregn-20-one sulfate; 3ββS, 3β-hydroxy-5β-pregn-20-one sulfate; 3αβHS, pregnanolone hemisuccinate; DMSO, dimethyl sulfoxide; DMEM, Dulbecco’s modified Eagle’s medium; 3αβ5S, 3α-hydroxy-5α-pregn-20-one sulfate; 3ββ5S, 3β-hydroxy-5α-pregn-20-one sulfate; 3αβ5HO, pregnanolone hemioxylate; 3αβ5HG, pregnanolone hemiglutarate; 3αβ5F, pregnanolone formate; PF, pregnanolone formate; PHO, pregnanolone hemioxylate; PHS, pregnanolone hemisuccinate; PHG, pregnanolone hemiglutarate.
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 (Corpechot et al., 1983), potentiates NMDA-induced currents (Wu et al., 1991; Bowlby, 1993), whereas pregnanolone sulfate (3α-hydroxy-5β-pregnan-20-one sulfate; 3α5βS) and 3β-hydroxy-5α-pregnan-20-one sulfate (3β5βS) inhibit NMDA-induced currents (Park-Chung et al., 1994; Yaghubi et al., 1998). Moreover, the steroid negative modulators 17β-estradiol (Weaver et al., 1997b) and pregnanolone hemisuccinate (3α5βS; 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 aqueous 1 N HCl. The organic phase was washed twice with 1 N NaCl (10 ml) and water (10 ml) and evaporated to dryness. The product was crystallized twice from a mixture of acetone and hexane. The hemiisuccinate esters were prepared as described above, but with 568 mg of oxalic acid instead of formic acid. The hemiglutarate esters of oxalic acid were prepared as described above, but with 568 mg of oxalic acid instead of formic acid. The hemiglutarate esters were prepared as described above, but with 568 mg of oxalic acid instead of formic acid. The hemioxalate esters were prepared as described above, but with 568 mg of oxalic acid instead of formic acid. The hemioxalate esters were prepared as described above, but with 568 mg of oxalic acid instead of formic acid. The hemioxalate esters were prepared as described above, but with 568 mg of oxalic acid instead of formic acid. The hemioxalate esters were prepared as described above, but with 568 mg of oxalic acid instead of formic acid.

**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 Pluronic 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, 1.5 mM MgCl2, 2.5 mM CaC12, 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, and 0.05% (w/v) Pluronic F-127.

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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 quantify 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 Ca²⁺ influx and cell death is expressed as the percentage of change, defined as (I/I’ − 1) × 100%, where I and I’ are the NMDA-induced responses in the absence and presence of modulator, respectively. All data are expressed as mean ± 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 Ca²⁺ 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 EC₅₀ 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 EC₅₀ 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 E₅₀ from 86 to 70% cell death without affecting the NMDA EC₅₀ (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 acidₐ receptor antagonist; Farrant and Webster, 1989) have no effect on NMDA-induced neuronal death (data not shown), arguing that the γ-aminobutyric acidₐ, 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-pregnan-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-pregnan-20-one sulfate (3β5αS), both potentiates 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 stereochchemistry 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 hemioxyxlate (3α5βHO), and pregnanolone hemiglutarate (3α5βHG) are about equally effective, inhibiting the NMDA-induced rise in [Ca²⁺]ᵢ by ~40% in primary hippocampal cultures, whereas the uncharged 3α5β and pregnanolone formate (3α5βF) have no significant effect on NMDA-induced Ca²⁺ accumulation (Fig. 3B). Similarly, 3α5βHO, 3α5βHS, and 3α5βHG are neuroprotective, reducing neuronal death caused by acute exposure to 30 μM NMDA by 35 ± 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; Bowley, 1993). To evaluate the role of the sulfate group at C₃, we examined the effects of pregnenolone, pregnenolone formate (PF), pregnenolone hemioxyxlate (PHO), pregnenolone hemisuccinate (PHS), and pregnenolone hemiglutarate (PHG; Fig. 4A).

Potentiation of NMDA-induced Ca²⁺ 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 3\(\alpha\)5\(\beta\)S 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 3\(\alpha\)5\(\beta\)S inhibits NMDA-induced currents in neurons maintained in primary culture (Park-Chung et al., 1994). It is interesting that, in acute treatments, 3\(\alpha\)5\(\beta\)S reduces both the NMDA EC\textsubscript{50} and E\textsubscript{max} for causing cell death, while only reducing the E\textsubscript{max} in chronic treatments. The reason for this difference is unclear,

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**Fig. 1.** 3\(\alpha\)5\(\beta\)S inhibits NMDA receptor function. A, 3\(\alpha\)5\(\beta\)S (100 \(\mu\)M) inhibits the 5 \(\mu\)M NMDA-evoked increase in [Ca\textsuperscript{2+}]\textsubscript{i}. Results are expressed as mean percentage of neuronal death ± S.E. of eight experiments. 3\(\alpha\)5\(\beta\)S or DMSO vehicle was present during the 15-min NMDA exposure only. Results are expressed as mean percentage of neuronal death ± S.E. of 16 (○; DMSO control) and 4 (●; 3\(\alpha\)5\(\beta\)S) experiments. Smooth curves were determined by nonlinear regression with the logistic equation applied to the pooled data (vehicle treated: \(E_{\text{max}} = 80\%\), EC\textsubscript{50} = 28 \(\mu\)M, \(n_H = 2.1\); 3\(\alpha\)5\(\beta\)S treated: \(E_{\text{max}} = 63\%\), EC\textsubscript{50} = 71 \(\mu\)M, \(n_H = 1.8\)). C, effect of 3\(\alpha\)5\(\beta\)S on the neuronal death produced by acute 30 \(\mu\)M NMDA exposure is dose dependent. Results are expressed as mean percentage of neuronal death ± S.E. of four experiments. Smooth curve was determined by nonlinear regression with the logistic equation applied to the pooled data (\(E_{\text{max}} = 97\%\) protection, EC\textsubscript{50} = 45 \(\mu\)M, \(n_H = 2.5\)). D, under chronic (16 h) exposure conditions, 3\(\alpha\)5\(\beta\)S (100 \(\mu\)M) reduces the NMDA efficacy but does not alter the affinity. 3\(\alpha\)5\(\beta\)S or DMSO vehicle was present during the 16 h NMDA exposure only. Results are expressed as mean percentage of neuronal death ± S.E. of 10 (DMSO control; ○) and 6 (3\(\alpha\)5\(\beta\)S; ●) experiments. Smooth curves were determined by nonlinear regression with the logistic equation applied to the pooled data (vehicle treated: \(E_{\text{max}} = 86\%\), EC\textsubscript{50} = 12 \(\mu\)M, \(n_H = 1.9\); 3\(\alpha\)5\(\beta\)S treated: \(E_{\text{max}} = 70\%\), EC\textsubscript{50} = 15 \(\mu\)M, \(n_H = 2.0\)). The break in the x-axis represents a change from linear to logarithmic scale. * statistically significant (\(P < .05\)) difference from NMDA control.
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+]i. 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. 3. C3 dicarboxylic acid esters of pregnanolone inhibit NMDA mediated Ca\textsuperscript{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 pregnane steroids inhibit the 5 μM NMDA-mediated Ca\textsuperscript{2+} influx in rat hippocampal cultures. Columns show mean percentage of reduction of the NMDA-induced rise in [Ca\textsuperscript{2+}]\textsubscript{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. *P < .05, significant decrease in NMDA-induced elevation of [Ca\textsuperscript{2+}]\textsubscript{i}. †P < .05, significantly less protection than 3αβHO, 3αβHS, or 3αβHG.

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 preg-5-ene steroids potentiate the 5 μM NMDA-mediated Ca\textsuperscript{2+} influx in rat hippocampal cultures. Columns show mean percentage of potentiation of the NMDA-induced rise in [Ca\textsuperscript{2+}]\textsubscript{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. *P < .05, significant increase in NMDA-induced elevation of [Ca\textsuperscript{2+}]\textsubscript{i}. †P < .05, significantly less potentiation than PHG. C, PHO, PHS, and PHG (100 μM) exacerbate NMDA-induced death of rat hippocampal neurons (*P < .05). Results are expressed as mean percentage of neuronal death ± S.E. with the number of experiments indicated in parentheses. †P < .05, significantly less protection than PHO, PHS, or PHG.
Steroid modulators of acute NMDA-induced neuronal death is through an interaction with the NMDA receptor. To determine whether steroid modulation of acute NMDA-induced cell death is correlated to modulation of NMDA-induced increases in $[\text{Ca}^{2+}]$, the steroid-mediated change (%) in excitotoxicity is plotted against the change (%) in NMDA-induced $\text{Ca}^{2+}$ influx. Steroid modulation of NMDA-induced cell death is strongly correlated to modulation of NMDA-induced increases in $[\text{Ca}^{2+}]$, ($r = 0.87$) 1, PS (Weaver et al., 1998); 2, PHS; 3, PHG; 4, PHO; 5, 3b5aS; 6, PF; 7, 3a5bF; 8, pregnenolone; 9, 3a5b; 10, 3a5bHO; 11, 3a5bHG; 12, 3a5bHS (Weaver et al., 1997a); and 13, 3a5bBS. Results are expressed as mean ± S.E. of at least three experiments.

**Importance of a Negatively Charged Group at C3.** To investigate the structural requirements for the C3 ester, a carboxylic acid chain-length series was synthesized in which the sulfate group of PS or 3a5bHS was replaced by a formate, hemioxylate, hemisuccinate, or hemiglutarate group. The parent compound, 3a5b, is without effect on NMDA-induced $\text{Ca}^{2+}$ influx and neuronal death, as is its uncharged formate derivative 3a5bF. However, the three charged derivatives, 3a5bHO, 3a5bHS, and 3a5bHG, reduce $\text{Ca}^{2+}$ accumulation and cell death of hippocampal neurons resulting from NMDA application.

The lack of activity of 3a5b and 3a5bF is consistent with our previous observation that 3a5bHS, but not the uncharged pregnenolone hemisuccinate methyl ester, inhibits NMDA-induced current in chick spinal cord neurons (Park-Chung et al., 1997). Therefore, inhibition of NMDA receptor function requires the presence of a negative charge adjacent to the C3 position, and it is not a nonspecific effect of the 3a5b steroid nucleus. A degree of tolerance for the geometry of the charged group at C3 is suggested by the fact that a range of lengths (from the relatively short hemioxylate group to the five-carbon hemiglutarate group) is able to confer inhibitory activity.

As with the pregnane steroids, the group esterified at the C3 hydroxyl group plays a crucial role in determining activity of preg-5-ene derivatives. The uncharged parent compound pregnenolone and its uncharged derivative PF do not potentiate NMDA-stimulated $\text{Ca}^{2+}$ accumulation or exacerbate NMDA-induced neuronal death. In contrast, the negatively charged PHO, PHS, and PHG potentiate the action of NMDA in both assays. Interestingly, PHO, although negatively charged, is significantly less effective than PHG in enhancing $\text{Ca}^{2+}$ accumulation by hippocampal neurons, suggesting that precise positioning of the negative charge is important for potentiation by preg-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 hemioxylate 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 3a5bHS is effective as a hypnotic, anticonvulsant, and analgesic, 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 preg-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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