Steroids Modulate N-Methyl-d-aspartate-Stimulated \([^{3}H]\)Dopamine Release from Rat Striatum via \(\sigma\) Receptors

SAMER J. NUWAYHID and LINDA L. WERLING

Department of Pharmacology, George Washington University Medical Center, Washington, DC

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ABSTRACT

Steroids have been proposed as endogenous ligands at \(\sigma\) receptors. In the current study, we examined the ability of steroids to regulate \(N\)-methyl-d-aspartate (NMDA)-stimulated \([^{3}H]\)dopamine release from slices of rat striatal tissue. We found that both progesterone and pregnenolone inhibit \([^{3}H]\)dopamine release in a concentration-dependent manner similarly to prototypical agonists, such as (+)-pentazocine. The inhibition seen by both progesterone and pregnenolone exhibits \(IC_{50}\) values consistent with reported \(K_{i}\) values for these steroids obtained in binding studies, and was fully reversed by both the \(\sigma_{1}\) antagonist 1-(cyclopropylmethyl)-4-[2-4\(^{\prime}\)fluorophenyl]-2-oxoethyl)piperidine HBr (DuP734) and the \(\sigma_{2}\) antagonist 1-\([4-[1-(4-fluorophenyl)-1-\(H\)-indol-3-yl]-1-buty1]spiro[iso-benzofuran-1(3\(H\))-4\(^{\prime}\)piperidine] (Lu28-179). Lastly, to determine whether a protein kinase C (PKC) signaling system might be involved in the inhibition of NMDA-stimulated \([^{3}H]\)dopamine release, we tested the PKC\(\beta\)-selective inhibitor 5,21:12,17-dimetheno-18\(\beta\)-dibenzo[\(i,o\)]pyrrolo[3,4-1]\(\[1,8\]\)diacyclohexadecine-18,20(19\(\beta\))-dione, 8-\([[(dimethylamin)\]methyl]-6,7,8,9,10,11-hexahydro-monomethanesulfonate \((9Cl)\) (LY379196) against both progesterone and pregnenolone. We found that LY379196 at 30 nM reversed the inhibition of release by both progesterone and pregnenolone. These findings support steroids as candidates for endogenous ligands at \(\sigma\) receptors.

Since their proposal in 1976 by Martin et al. (1976), \(\sigma\) receptors have been characterized pharmacologically in bioassays and radioligand binding studies. \(\sigma\) receptors bind a wide array of drugs from various classes, including benzomorphans, guanidines, morphinans, antipsychotics, and cocaine. However, none of these drugs is endogenous to the brain or in cells in culture. To establish a relevance of \(\sigma\) receptors to physiological function, it is important to identify an endogenous ligand.

Steroids were originally proposed as endogenous ligands at \(\sigma\) receptors by Su et al. (1988). Swartz et al. (1989) questioned this proposal because they assumed steroids could not be produced in the brain, and the concentration of steroids crossing the blood-brain barrier would not suffice to occupy \(\sigma\) receptors. Interest in steroid-\(\sigma\) receptor interactions was re-vived when studies showed that steroids mediated many effects through a nongenomic mechanism (Falkenstein et al., 2000) and that steroids were synthesized in the brain (Hu et al., 1987; Jung-Testas et al., 1989; Guarneri et al., 2000), along with pharmacological studies on regulation of transmitter release (Monnet et al., 1995) and responses in hippocampal neurons (Bergeron et al., 1996) to steroids. The steroids the Su group found that competed best for \([^{3}H]\)SKF10,047 at \(\sigma_{1}\) sites were progesterone, deoxycorticosterone, and testosterone. More recently, McCann and Su (1994) examined steroid competition at \(\sigma_{2}\) sites and found both progesterone and testosterone had an affinity for \(\sigma_{1}\) and \(\sigma_{2}\) subtypes.

Monnet et al. (1995) described the modulation of NMDA-stimulated \([^{3}H]\)norepinephrine release by progesterone, dehydroepiandrosterone sulfate (DHEA S), and pregnenolone sulfate (PREG S) in rat hippocampal slices. The effects were blocked by \(\sigma\) receptor antagonists and progesterone acted as an antagonist to DHEA S and PREG S. Most recently, Meyer et al. (2002) showed that PREG S enhances glutamate re-

ABBREVIATIONS: NMDA, \(N\)-methyl-d-aspartate; DHEA S, dehydroepiandrosterone sulfate; PREG S, pregnenolone sulfate; DHEA, dehydroepiandrosterone; PKC, protein kinase C; PLC, phospholipase C; MKB, modified Krebs-HEPES buffer; S1, first stimulus; SII, inter stimulus interval; S2, second stimulus; LY379196, 5,21:12,17-dimetheno-18\(\beta\)-dibenzo[\(i,o\)]pyrrolo[3,4-1]\(\[1,8\]\)diacyclohexadecine-18,20(19\(\beta\))-dione, 8-\([[(dimethylamin)\]methyl]-6,7,8,9,10,11-hexahydro-monomethanesulfonate \((9Cl)\); BD1063, 1\([2-[3,4\,-dichlorophenyl]ethyl]-4\)-methylpiperazine; DuP734, 1\-(cyclopropylmethyl)-4-\([2-4\(^{\prime}\)fluorophenyl]-2-oxoethyl)piperidine HBr; Lu28-179, 1-\([4-[1-(4-fluorophenyl)-1\(H\)-indol-3-yl]-1-buty1]spiro[iso-benzofuran-1(3\(H\))-4\(^{\prime}\)piperidine]; GF109203x, 3\-[3-(dimethylamino)propyl]-1\(H\)-indol-3-yl]-1\(H\)-pyrrolo-2,5-dione; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N,N' \(\cdot\) tetraacetic acid; SKF10,047, \(n\)-allylnormetazocine; BD737, 1,5\(\beta\)-[(-)-cis-N\(\cdot\)-[2,3\,-dichlorophenyl]ethyl]-N-methyl-2\(\cdot\)-pyrrolidinyl]-cyclohexylamine; U73122, 1\-[6\-[17\(-b\,-3\)-methoxyestra-1,3,5(10)-tri-en-17\(\beta\)]amino]hexyl]-1\(H\)-pyrrolo-2,5-dione.
lease from hippocampal slices via a σ1-like receptor. In studies reviewed by Bastianetto et al. (1999) and Maurice et al. (1999), PREG S, DHEA S, and allotetrahydroxyxycorticosterone show antistress, anxiolytic, and antiinflammatory activity that is blocked by σ antagonists, as well as antisense oligonucleotides to σ1 receptors (Maurice et al., 2001). Taking all these studies in consideration, steroids at this time are likely candidates for endogenous σ receptor ligands.

Gonzalez-Alvear and Werling (1994) first demonstrated regulation of NMDA-stimulated [3H]dopamine release from rat striatum by σ receptor ligands, including (+)-pentazocine, (+)-SKF10,047, and BD737. The inhibition produced by low concentrations of these ligands was reversed by the σ1 antagonist DuP734 (Gonzalez-Alvear and Werling, 1994, 1995). A second phase of inhibition produced by higher concentrations of (+)-pentazocine was reversed by nonsubtype-selective σ receptor antagonists, indicating the participation of σ2 receptors. Studies by Izenwasser et al. (1998) revealed that amphetamine-stimulated [3H]dopamine release can be modulated by σ2 receptor agonists and antagonists in vitro. In our current study, we examined the ability of steroids to regulate NMDA-stimulated [3H]dopamine release from rat striatal slices via σ receptors. If steroids are indeed the endogenous ligand for σ receptors, they should affect neurotransmitter release and signaling similarly to prototypical σ ligands. We have previously demonstrated that σ1 agonist-mediated inhibition of NMDA-stimulated [3H]dopamine release is mediated by a PKC signaling system, likely involving the β isoform. σ receptor regulation of release is abolished by pretreatment with phorbol 12-myristate 13-acetate, as well by treatment with a PKCβ inhibitor (Nuwahyid and Werling, 2003). Therefore, we tested whether the same PKC pathway is involved in regulation of stimulated dopamine release by steroids via σ receptors by using a PKCβ inhibitor.

Materials and Methods

Drugs and Reagents. The following chemicals were kindly provided by or obtained from the following sources: domperidone and nomifensine (Sigma/RBI, Natick, MA); 1-ascorbic acid, pregnenolone, testosterone, progesterone, dihydroxycorticosterone, cholesterol, and mifepristone (Sigma-Aldrich, St. Louis, MO); [3H]dopamine (Amersham Biosciences Inc., Piscataway, NJ); LY379196 (gift of Dr. Robin Bowman, Eli Lilly & Co., Indianapolis, IN); trilostane (gift of Dr. J. Puddefoote; Queen Mary and Westfield College, London, UK); DuP734 (DuPont Merck Pharmaceutical Co., Wilmington, DE); and Lu28-179 (H. Lundbeck, Copenhagen, Denmark).

Measurement of Stimulated [3H]Dopamine Release from Striatal Slices. All experiments were carried out in accordance with the guidelines and the approval of the George Washington University Institutional Animal Use and Care Committee. Male Sprague-Dawley rats (Hilltop Lab Animals, Scottsdale, PA), weighing 200 to 225 g, were sacrificed by decapitation, and brains removed to ice. Striata were dissected, chopped in two planes at right angles into 250 × 250 μm strips with a Sorvall T-2 tissue sectioner, and suspended in modified Krebs-HEPES buffer (MKB; 127 mM NaCl, 5 mM KCl, 1.3 mM NaH₂PO₄, 2.5 mM CaCl₂, 15 mM HEPES, 10 mM glucose, pH adjusted to 7.4 with NaOH) by titration through a plastic pipette. Buffers were oxygenated throughout the experiments and brain slices were kept at a constant temperature of 37°C. After three washes in MKB, tissue was resuspended in 20 ml of MKB and incubated for 30 min with 0.1 mM ascorbic acid and 15 mM [3H]dopamine. Tissue was then washed twice with 20 ml of MKB and once in 20 ml of MKB containing 10 μM nomifensine and 1 μM domperi-
done. These drugs were included in all subsequent steps to prevent reuptake of and feedback inhibition by the released [3H]dopamine. Tissue was suspended a final time in 7.5 ml of MKB, containing 10 μM nomifensine and 1 μM domperidone, and oxygenated in 275-μl aliquots between glass fiber discs into chambers of a superfusion apparatus (Brandel, Inc., Gaithersburg, MD). MKB was superfused over tissue at a rate of 0.6 ml/min. A low stable baseline release of approximately 1.3%/2 min collection interval was established over a 30-min period. Tissue was then stimulated by a 2-min exposure to 25 μM NMDA (S1). The mean fractional release (percentage) produced in the S1 stimulus interval was 11.9 ± 1.2%. Infow was then returned to nonstimulating buffer during a 10-min interstimulus interval (ISI). If a steroid, σ antagonist, cholesterol, mifepristone, or trilostane was being tested, it was included at this time. The inclusion of σ antagonist drug in the buffer did not significantly affect basal release (per 2-min collection interval: no antagonist 1.3 ± 0.33, n = 3; 100 nM DuP734, 1.2 ± 0.24, n = 3; 1 μM Lu28-179 1.4 ± 0.14, n = 3). Neither did inclusion of steroid affect basal release significantly (progesterone, 1.3 ± 0.24, n = 4; pregnenolone, 1.6 ± 0.09, n = 3) . Tissue was then exposed to a second stimulus (S2) identical to the first except in the presence of a steroid, trilostane, mifepristone, or cholesterol, as appropriate. In the experiments testing the PKC inhibitor LY379196, the drug was present throughout S1, ISI, and S2. Infow was once again returned to nonstimulating buffer before extraction of the remaining radioactivity in the tissue by a 45-min exposure to 0.2 N HCl at a reduced flow rate. Superfusates were collected at 2-min intervals in scintillation vials with the glass fiber filter discs and tissue collected into the final vials. Released radioactivity was determined by liquid scintillation spectroscopy.

All data were statistically analyzed as ratios (S2/S1) before conversion to percentage of control values for presentation. The ratio of S2/S1 in the absence of any test drug was 0.54 ± 0.07 (n = 10). An enhancement by test drug would result in a higher ratio and an inhibition in a lower ratio. In this way, differences in response between tissue samples are taken into account and therefore, do not affect the comparison of treatments. In the results, data are expressed as radioactivity released above baseline during the collection interval as a fraction of the total radioactivity in the tissue at the beginning of the collection interval (fractional release, percentage) or as a percentage of the radioactivity released by the control stimulus (percentage of control-stimulated release). Data are presented as a percentage of control-stimulated release for facilitation of comparison across experiments. Under the experimental conditions used, the released radioactivity has the time course expected for comparably dopamine (Werling et al., 1988). All statistical analyses were performed by one-way factorial analysis of variance with post hoc Dunnett’s. Statistical significance is considered at p < 0.05.

Results

To examine the possibility that steroids could act similarly to identified agonists or antagonists at σ receptors, we tested several steroids for their ability to regulate NMDA-stimulated [3H]dopamine release via σ receptors from rat striatal tissue. We first tested single concentrations of progesterone, pregnenolone, DHEA, and testosterone to determine whether they had any effect on NMDA-stimulated [3H]dopamine release. As seen in Fig. 1, neither DHEA at 10 μM (Kᵢ at σ receptors = 3.7 μM, undifferentiated for subtype; Klein and Musacchio, 1994) nor testosterone at 3 or 10 μM (Kᵢ = 1 μM, undifferentiated for subtype; Su et al., 1988) showed any significant difference from control-stimulated release.
Progesterone has a $K_i$ value of 270 nM at $\sigma$ receptors, undifferentiated for subtype (Su et al., 1988). The $IC_{50}$ for inhibition of release was similar to this value, lying between 100 and 300 nM. Progesterone significantly inhibited release at 300 nM and 1 $\mu$M. At 1 $\mu$M, the highest concentration tested, progesterone inhibited release approximately 30%, similar to the maximum inhibition produced by (+)-pentazocine that was attributable to $\sigma$ receptors (Gonzalez-Alvear and Werling, 1994). To confirm whether the inhibition by progesterone was mediated through $\sigma$ receptors, we tested the $\sigma_1$ receptor-selective antagonist DuP734 (100 nM) and the $\sigma_2$ receptor-selective antagonist Lu28-179 (1 nM) against 1 $\mu$M progesterone (Fig. 2A). Both DuP734 and Lu2-1798 completely reversed the inhibition of release by progesterone to values slightly above control-stimulated release. The elevation above control is not significant for Lu2-179, and although the value for DuP734 achieves statistical significance as differing for NMDA alone, is associated with a high error value.

Next, we constructed a concentration-response curve for pregnenolone. As seen in Fig. 2B, the $IC_{50}$ value for inhibition of release was between 300 nM and 1 $\mu$M. Pregnenolone at 3 $\mu$M inhibited release approximately 25%. As with progesterone, we also tested pregnenolone (3 $\mu$M) with both the $\sigma_1$ antagonist DuP734 (100 nM) and the $\sigma_2$ antagonist Lu28-179 (1 nM). Both DuP734 and Lu2-1798 completely reversed the inhibition by pregnenolone (Fig. 2B) to levels not different from control-stimulated release.

Because DHEA and testosterone have affinity for $\sigma$ receptors, but did not show any inhibition of NMDA-stimulated $[^3H]$dopamine release, we tested whether they might behave as $\sigma$ antagonists in our assay. We tested DHEA and testosterone in combination with both progesterone and pregnenolone to examine whether they reversed the inhibition of NMDA-stimulated $[^3H]$dopamine release seen by both progesterone and pregnenolone. Neither DHEA (10 $\mu$M) nor testosterone (3 $\mu$M) had any effect on the inhibition of $[^3H]$dopamine release seen by either progesterone (Fig. 3A) or pregnenolone (Fig. 3B).

To determine whether PKC$\beta$ is involved in the inhibition of NMDA-stimulated $[^3H]$dopamine release, we conducted dose-response curves for the PKC$\beta$-selective inhibitor LY379196 against both progesterone and pregnenolone. We have previously shown that LY379196 blocked (+)-pentazocine-mediated inhibition of NMDA-stimulated $[^3H]$dopamine release in a concentration-dependent manner (Nuwayhid and Werling, 2003). LY379196 has $K_i$ values at $\beta_1$ and $\beta_2$ isozymes of 18 and 16 nM, and all other isozymes, including PKC$\alpha$, PKC$\beta$, PKC$\gamma$, PKCe, and PKC$\eta$ >300 nM (Louis Vignalii, personal communication). As seen in Fig. 4A, LY379196 completely reversed the inhibition of release by 1 $\mu$M progesterone at both 30 and 100 nM. At 30 nM, LY379196 completely reversed the inhibition of release by 3 $\mu$M pregnenolone (Fig. 4B).

Progesterone and pregnenolone are synthesized from cholesterol, as demonstrated in neural cells in culture (Guaneri et al., 2000). We therefore tested cholesterol to determine whether it, as the parent compound, displayed any effects on
NMDA-stimulated $[^{3}H]$dopamine release. Cholesterol (25 μM) did not affect NMDA-stimulated release of $[^{3}H]$dopamine (B). Release of $[^{3}H]$dopamine from slices of rat striatal tissue was stimulated by 25 μM NMDA alone or 25 μM NMDA in the presence of antagonist steroid testosterone (3 μM) or DHEA (10 μM). Data are expressed as percentage of control NMDA-stimulated release. *, significantly different from NMDA alone at $p < 0.05$. Neither the inclusion of testosterone nor DHEA significantly changed the inhibition produced by progesterone or pregnenolone alone. $n = 3$ independent experiments in which each treatment was tested in triplicate. Note breaks in y-axes.

Last, to verify the effects seen by pregnenolone on NMDA-stimulated $[^{3}H]$dopamine release were attributed to pregnenolone and not dependent upon its conversion to progesterone or other metabolite, we tested pregnenolone in the presence of 20 μM trilostane. Trilostane is an inhibitor of $3\beta$-hydroxysteroid dehydrogenase, an enzyme that converts pregnenolone to progesterone ($K_i = 50$ nM; Takahashi et al., 1990). As seen in Fig. 6, inhibition of $[^{3}H]$dopamine release by 3 μM pregnenolone in the presence of trilostane was not significantly different compared with the inhibition of $[^{3}H]$dopamine release by 3 μM pregnenolone alone. We also verified that progesterone was acting through $\sigma$ receptors and not progesterone receptors by testing 1 μM progesterone in the presence of 10 μM mifepristone, a progesterone receptor antagonist. As seen in Fig. 7, there was no difference in the inhibition seen by 1 μM progesterone or 3 μM pregnenolone without PKC inhibitor at $p < 0.05$, $n = 3$ independent experiments in which each treatment was tested in triplicate. Note breaks in y-axes.

Discussion

The identity of the endogenous ligand for $\sigma$ receptors has been equivocal. Su et al. (1988) found that progesterone, deoxycorticosterone, and testosterone competed for $[^{3}H]$SKF10,047 at $\sigma_1$ sites in guinea pig brain tissue and proposed steroids as the endogenous ligands. Several studies have now demonstrated...
that steroids are synthesized in the brain (Hu et al., 1987; Jung-Testas et al., 1989; Guarneri et al., 2000).

Steroids exhibit genomic and nongenomic effects (Ruppert and Holsboer, 1999). In the current study, we examined presumably nongenomic effects of steroids on NMDA-stimulated \[^3H\]dopamine release. The effects on regulation of dopamine release occur within a relatively short time frame because the length of exposure of tissue to steroid is 12 min, not likely sufficient to produce changes in protein expression. If steroids are endogenous ligands for \( \sigma \) receptors they should behave similarly to prototypic ligands, such as \((+)-\)pentazocine.

We found that both progesterone and pregnenolone inhibited NMDA-stimulated \[^3H\]dopamine release. Both inhibited release in a concentration-dependent manner, with a maximum of about 25 to 30%, similar to the inhibition seen by \((+)-\)pentazocine in studies by Gonzalez-Alvear and Werling (1994, 1995). The IC\textsubscript{50} value of progesterone (300 nM) for inhibition of NMDA-stimulated \[^3H\]dopamine release was similar to its \( K_i \) value of 270 nM in competing for binding to \( \sigma \) receptors (Su et al., 1988). Pregnenolone showed an IC\textsubscript{50} value in the range of 300 nM to 1 \( \mu \)M. A \( K_i \) value at \( \sigma \) receptors in brain tissue has not been reported, but we found that pregnenolone competed for \( \sigma_1 \) binding with a \( K_i \) value of 980 \( \pm \) 340 nM in SH-SY5Y cells (Werling, 2002). Su et al. (1988) reported a \( K_i \) value of 3.2 \( \mu \)M for pregnenolone sulfate binding to \( \sigma_1 \) receptors. These findings support that pregnenolone acts via \( \sigma \) receptors to inhibit NMDA-stimulated \[^3H\]dopamine release.

The hypothesis that progesterone and pregnenolone act as \( \sigma \) agonists in our assay was confirmed by the action of \( \sigma \) antagonists. The inhibition of NMDA-stimulated \[^3H\]dopamine release by 1 \( \mu \)M progesterone and 3 \( \mu \)M pregnenolone was fully reversed by the \( \sigma_1 \) antagonist DuP734 (\( K_i = 10 \) nM) (Culp et al., 1992) at 100 nM and the \( \sigma_2 \) antagonist Lu28-179 (\( K_i = 0.12 \) nM) (Moltzen et al., 1995) at 1 nM. The reversal seen by 100 nM DuP734 was somewhat above control, although associated with a relatively high error determination. It is possible that there is antagonism of tone exerted by endogenous steroids. However, the antagonists had no effect on basal release, which argues against this possibility. The action seen by both DuP734 and Lu28-179 suggest that the inhibition of \[^3H\]dopamine release is mediated through both \( \sigma_1 \) and \( \sigma_2 \) receptor subtypes. This is a contrast to our findings (Gonzalez-Alvear and Werling, 1994, 1995) in which components of \((+)-\)pentazocine-mediated inhibition were clearly attributable to either \( \sigma_1 \) or \( \sigma_2 \) receptors based on reversal by subtype-selective antagonists. However, in our assays examining the effects of \( \sigma_2 \) agonists on amphetamine-stimulated \[^3H\]dopamine release from rat striatal slices, we found sim-
ilar antagonism of effect by $\sigma_1$ and $\sigma_2$ antagonists (Liu et al., 2001) to that seen for the current steroid experiments. The $\sigma_1$ and $\sigma_2$ receptors are relatively small ($\sigma_1 = 28$ kDa, Hannen et al., 1996; $\sigma_2 = 22$ kDa, Hellewell and Bowen, 1990). Perhaps upon activation of $\sigma$ receptor subtypes by progesterone and pregnenolone the receptors interact with each other, which in turn inhibits [$^3$H]dopamine release. In this situation, competing off the steroid agonist with either $\sigma_1$ or $\sigma_2$ antagonists would prevent regulation of dopamine release. Reports in which $\sigma$ receptor protein was purified and detected via photoaffinity labeling (Schuster et al., 1995) or with antibody selective for $\sigma$ receptor (Hanner et al., 1996), show a band with a molecular weight of approximately $60$ kDa, which could be a dimer of $\sigma_1$ and/or $\sigma_2$ receptors. Neuroactive steroids are known to modulate the actions of GABA$_\alpha$ and NMDA receptors (Ruppert and Holsboer, 1999). Although it cannot be absolutely excluded that the effects of the steroids observed in the current study involve actions via one of these receptors, the regulation of dopamine release by the steroids is presumably via actions at the dopaminergic nerve terminal, because $\sigma$ receptors regulating dopamine release have been localized to that location (Gonzalez-Alvear and Werling, 1995). Regardless of whether GABA$_\alpha$ or NMDA receptors contribute in some way to the overall response, the effects of progesterone and pregnenolone are both completely reversed in the current study by $\sigma$ receptor antagonists.

The concentrations of DHEA and testosterone tested were chosen based on their reported $K_i$ values from binding studies. Although both competed for $\sigma$ receptor binding (Su et al., 1988; Klein and Musacchio, 1994), our data do not indicate that the $\sigma$ receptor subtype(s) involved in the regulation of dopamine release are sensitive to these steroids. Neither showed an inhibition of stimulated dopamine release, and when tested for potential $\sigma$ antagonist activity, neither reversed the inhibition of [$^3$H]dopamine release by progesterone or pregnenolone. DHEA and testosterone may bind to $\sigma$ receptors but do not have either agonist or antagonist properties in our system. It is possible that higher or lower concentrations could have produced effects, although this would not be predicted based on reported affinities at $\sigma$ receptors. Further confirming that $\sigma$ agonist properties are conferred only upon specific steroids, and not the heterocyclic steroidal structure in general, was the finding that cholesterol, the parent compound from which steroids are derived, had no effect on NMDA-stimulated [$^3$H]dopamine release.

We confirmed that the effects displayed by pregnenolone were due to pregnenolone itself and not its metabolite progesterone, using trilostane, an inhibitor of 3β-hydroxysteroid dehydrogenase enzyme that converts pregnenolone into progesterone. Therefore, both progesterone and pregnenolone seem to act as $\sigma$ receptors agonists in the regulation of dopamine release.

Monnet et al. (1995) showed that DHEA S potentiated [$^3$H]norepinephrine release from rat hippocampal slices and this response was reversed by both $\sigma$ antagonists haloperidol and BD1063. In a later article (Monnet et al., 1996), they also showed regulation of norepinephrine release by other steroids, but with a different pharmacology than ours. Our data show regulation of NMDA-stimulated [$^3$H]dopamine release by steroids is inhibitory. This could be due to the difference in the neurotransmitter studied or other experimental variables. We have previously shown that $\sigma$ receptors that regulate dopamine release from striatum are located on dopaminergic nerve terminals, but those regulating norepinephrine release are not. Therefore, the strength of the stimulus and the circuitry involved in hippocampus is likely to be much more complex. Monnet et al. (1995) used a concentration of NMDA 4 times that used by us in the current experiment and in our previous experiments on norepinephrine. The higher NMDA concentration could activate multiple other neurons upon which sigma receptors are located. Monnet et al. (1995) identified that the effects observed for some $\sigma$ agonists were mediated by different populations of $\sigma$ receptor subtypes depending upon the agonist used. It seems that our responses are due to activation of only $\sigma_1$ and $\sigma_2$ subtypes only as identified by selective antagonists.

Other groups have also reported effects of steroids that were mediated by $\sigma$ receptors. PREG S increase spontaneous glutamate release via activation of a presynaptic GABA$_\alpha$-coupled $\sigma$ receptor (Meyer et al., 2002). Because steroid sulfotransferases and sulfatases are present in the central nervous system (Rajkowski et al., 1997), both sulfated and nonsulfated forms could be present in the brain and modulate neurotransmitter release. However, the permeability of steroids is reduced by the addition of sulfate; if $\sigma$ receptors are intracellular, as suggested by McCann and Su (1994) and Vilner and Bowen (2000), the actions of sulfated steroids would be limited. Unsulfated steroids would have to enter the cell and be subsequently sulfated to become active. It is also possible that sulfated steroids could be concentrated and stored in vesicles (Gibbs and Farb, 2000). However, the weak activities of sulfatases and sulfotransferases make the conversion of steroids in vivo doubtful (Baulieu and Robel, 1996).

Studies have now implicated the PKC signaling system in $\sigma$ receptor mediated processes. GF109203x, a PKC inhibitor, abolished $\sigma_2$ receptor-mediated regulation of dopamine transporter activity (Derbez et al., 2002). Morin-Surin et al. (1999) showed a parallel translocation of $\sigma$ receptors and PKCβ1 and PKCβII from the cytosol to the plasma membrane upon $\sigma$ agonist application. They also showed a time-dependent desensitization in the ability of (+)-pentazocine to reduce the firing rate in rat hypoglossal neurons. This desensitization may have been attributed to the desensitization of PKC itself. In our studies, LY379196, a PKCβ-selective inhibitor, blocked the inhibition of [$^3$H]dopamine release produced by (+)-pentazocine (Nuwayhid and Werling, 2003). U73122, a PLC inhibitor, also blocked (+)-pentazocine-mediated regulation. This suggests that in order for (+)-pentazocine to exert its inhibition of dopamine release the PLC/PKC system must be intact. PKC activation has also been linked to $\sigma$ inhibition of NMDA-stimulated $Ca^{2+}$ changes in cerebellar granular cells (Snell et al., 1994).

In the current study, we determined that the inhibition of NMDA-stimulated [$^3$H]dopamine release by steroids is dependent upon PKC activation. Steroids are known to interact with the PKC pathway. Progesterone can rapidly stimulate phosphatidylinositol bisphosphate hydrolysis, leading to the formation of diacylglycerol and inositol triphosphate, presumably due to the action of $Ca^{2+}$-dependent PKC (Thomas and Meisel, 1989). Estrogen increased inositol triphosphate concentrations and stimulated PKCα levels in membrane fraction of HEPG2 cells through a nonclassical steroid mechanism (Marino et al., 1998). Last, in studies by Condiffe et al. (2001), 17-β-estradiol regulated Cl$^-$ secretion in rat co-
Ionic epithelium, and regulation is blocked by the chelation of Ca\textsuperscript{2+} with BAPTA or the PKC inhibitor chelerythrine. In our study, steroids exhibited the same effects of (+)-pentazocine in the presence of LY379196, an inhibitor of PKC\textbeta isozymes. This reinforces the possibility that steroids have nongenomic actions and can signal through PLC/PKC via their actions at \sigma receptors.

In conclusion, both progesterone and pregnenolone have been identified as \sigma receptor agonists in the modulation of NMDA-stimulated [\textsuperscript{3}H]dopamine release. We found them to behave similarly to prototypic ligands, such as (+)-pentazocine. In addition, we have shown that the inhibition of NMDA-stimulated [\textsuperscript{3}H]dopamine release mediated by progesterone and pregnenolone involves a PKC signaling system most likely to involve the PKC\beta isozyme. Our findings in this study further support steroids as candidates for endogenous ligands at \sigma receptors.

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Address correspondence to: Dr. Linda L. Werling, Department of Pharmaco-