

Steroids modulate NMDA-stimulated [³H]dopamine release from rat striatum via sigma
receptors*

Samer J. Nuwayhid and Linda L. Werling

Department of Pharmacology

The George Washington University Medical Center

Washington, DC 20037

Running title: Steroids modulate dopamine release via sigma receptors

Key words: progesterone, pregnenolone, LY379196, dopamine release

Author for Correspondence:

Linda L. Werling, Ph.D.

Department of Pharmacology

The George Washington University

2300 Eye Street, NW

Washington, DC 20037

Telephone: 202-994-2918

Fax: 202-994-2870

E-mail: phllw@gwumc.edu

Number of text pages:

Number of tables: 0

Number of figures: 7

Number of references: 39

Number of words in:

Abstract: 154

Introduction: 574

Discussion: 1692

Abbreviations

ANOVA, analysis variance; BD1063, 1[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine; DAG, diacylglycerol; DAT, dopamine transporter; DHEA, dehydroepiandrosterone; DHEA S, dehydroepiandrosterone sulfate; DuP734, 1-(cyclopropylmethyl)-4-2'-4''fluorophenyl)-2'oxoethyl)piperidine HBr; ER, endoplasmic reticulum; GF109203x, 3[1-[3-(dimethylamino)propyl]-1H-indol-3-yl]-1-H-pyrrole-2,5-dione; IP₃, inositol triphosphate; ISI, inter stimulus interval; Lu28-179, 1'-[4-[1-(4-fluorophenyl)-1-*H*-indol-3-yl]-1-butyl]spiro[isobenzofuran-1(3H), 4' piperidine]; LY379196, 5,21:12,17-dimetheno-18H-dibenzo[*i,o*]pyrrolo[3,4-*l*][1,8]diacyclohexadecine-18,20(19H)-dione,8-[(dimethylamino)methyl]-6,7,8,9,10,11-hexahydro-monomethanesulfonate (9Cl); MKB, modified Krebs-HEPES buffer; NMDA, N-methyl-D-aspartate; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 2-myristate 13-acetate; (+)pent, (+)pentazocine; preg, pregnenolone; prog, progesterone; σ , sigma; S1, first stimulus; S2, second stimulus; test, testosterone

Recommended section assignment: Neuropharmacology

Abstract

Steroids have been proposed as endogenous ligands at sigma (σ) 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 σ_1 antagonist 1-(cyclopropylmethyl)-4-2'-4''fluorophenyl)-2'oxoethyl)piperidine HBr (DuP734) and the σ_2 antagonist 1'-[4-[1-(4-fluorophenyl)-1-*H*-indol-3-yl]-1-butyl]spiro[iso-benzofuran-1(3H), 4'piperidine] (Lu28-179). Lastly, to determine if a PKC signaling system might be involved in the inhibition of NMDA-stimulated [3 H]dopamine release we tested the PKC β -selective inhibitor, 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 σ receptors.

Since their proposal in 1976 by Martin et al., sigma receptors (σ) 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. In order to establish a relevance of σ receptors to physiological function, it is important to identify an endogenous ligand.

Steroids were originally proposed as endogenous ligands at σ 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 σ receptors. Interest in steroid- σ receptor interactions was revived when studies showed that steroids mediated many effects through a non-genomic 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., 1995) to steroids. The steroids the Su group found that competed best for [3 H]SKF10,047 at σ_1 sites were progesterone, deoxycortisone and testosterone. More recently McCann and Su (1994) examined steroid competition at σ_1 sites and found both progesterone and testosterone had an affinity for σ_1 and σ_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 σ 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 release from hippocampal slices via a σ_1 -like receptor.

In studies reviewed by Bastianetto et al. (1999) and Maurice et al. (1999), PREG S, DHEA S and allotetrahydrocorticosterone show antistress, anxiolytic and anti-amnesiac activity that are blocked by σ antagonists, as well as antisense oligonucleotides to σ_1 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 [3 H]dopamine release from rat striatum by σ receptor ligands, including (+)pentazocine, (+)SKF-10,047 and BD737. The inhibition produced by low concentrations of these ligands was reversed by the σ_1 antagonist DuP734 (Gonzalez-Alvear and Werling, 1994, 1995). A second phase of inhibition produced by higher concentrations of (+)pentazocine was reversed by non-subtype-selective σ receptor antagonists, indicating the participation of σ_2 receptor. Studies by Izenwasser et al. (1998) revealed that amphetamine-stimulated [3 H]dopamine release can be modulated by σ_2 receptor agonists and antagonists in vitro. In our current study, we examined the ability of steroids to regulate NMDA-stimulated [3 H]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 σ_1 agonist-mediated inhibition of NMDA-stimulated [3 H]dopamine release is mediated by a PKC signaling system, likely involving the β isoform. Sigma receptor regulation of release is abolished by pretreatment with PMA, as well by treatment with a PKC β or a PLC inhibitor (Nuwayhid 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 PKC β inhibitor.

Methods

Drugs and reagents

The following chemicals were kindly provided by or obtained from the following sources: domperidone, nomifensine, Research Biochemicals International (Natick, MA); L-ascorbic acid, pregnenolone, testosterone, progesterone, dihydroisoandrosterone, cholesterol, mifepristone, Sigma Chemical Company (St. Louis, MO); [³H]dopamine, Amersham Corp. (Arlington Heights, IL); LY379196, gift of Dr. Robin Bowman, Eli Lilly and Company (Indianapolis, IN); trilostane, gift of Dr. J. Puddefoote (Queen Mary and Westfield College, London); DuP734, Dupont Merck; Lu-28-179, H. Lundbeck (Copenhagen).

Measurement of stimulated [³H]DA 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 200-225 g were sacrificed by decapitation, and brains removed to ice. Striata were dissected, chopped in two planes at right angles into 250 x 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. Following three washes in MKB, tissue was resuspended in

20 ml MKB and incubated for 30 min with 0.1 mM ascorbic acid and 15 nM [³H]dopamine. Tissue was then washed twice with 20 ml MKB and once in 20 ml MKB containing 10 μM nomifensine and 1 μM domperidone. 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 MKB, containing 10 μM nomifensine and 1 μM domperidone, and distributed in 275 μl aliquots between glass fiber discs into chambers of a BRANDEL superfusion apparatus (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 (%) produced in the S1 stimulus interval was 11.9 ± 1.2 %. Inflow was then returned to non-stimulating buffer during a 10 min interstimulus interval (ISI). If a steroid, σ antagonist, cholesterol, mifepristone or trilostane were being tested it was included at this time. The inclusion of σ antagonist drug in the buffer did not significantly affect basal release (per two min collection interval: no antagonist 1.3 ± 0.33, N = 3; 100 nM DuP734 1.2 ± 0.24, N = 3; 1 nM 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. Inflow was once again returned to non-stimulating buffer before extraction of the remaining radioactivity in the tissue by a 45 min exposure to 0.2N 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 % 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, %) or as a percentage of the radioactivity released by the control stimulus (% control stimulated release). Data are presented as % control stimulated release for facilitation of comparison across experiments. Under the experimental conditions used, the released radioactivity has been shown to be primarily dopamine (Werling et al., 1988). All statistical analyses were performed by one-way factorial ANOVA 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 if they had any effect on NMDA-stimulated [^3H]dopamine release. As seen in Figure 1, neither DHEA at 10 μM (K_i at σ receptors = 3.7 μM , undifferentiated for subtype; Klein and Musacchio, 1994) nor testosterone at 3 or 10 μM (K_i = 1 μM , undifferentiated for subtype; Su et al., 1988) showed any significant difference from control stimulated release.

In preliminary experiments, progesterone at 3 μM and pregnenolone at 1 μM showed an inhibition of NMDA-stimulated [^3H]dopamine release. We then constructed concentration-response curves for both these steroids. As seen in Figure 2A, progesterone inhibited NMDA-stimulated [^3H]dopamine release in a concentration dependent matter. Progesterone has a K_i of 270 nM at σ receptors, undifferentiated for subtype (Su, et al., 1988). The IC_{50} for inhibition of release was similar to this value, lying between 100 nM and 300 nM. Progesterone significantly inhibited release at 300 nM and 1 μM . At 1 μM , the highest concentration tested, progesterone inhibited release approximately 30%, similar to the maximum inhibition produced by (+)pentazocine that was attributable to σ receptors (Gonzalez-Alvear and Werling, 1994). In order to confirm if the inhibition by progesterone was mediated through σ receptors, we tested the σ_1 receptor-selective antagonist DuP734 (100 nM) and the σ_2 receptor-selective antagonist Lu28-179 (1 nM) against 1 μM progesterone (Figure 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 Lu28-179, 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 Figure 2B, the IC_{50} for inhibition of release was between 300 nM and 1 μ M. Pregnenolone at 3 μ M inhibited release approximately 25%. As with progesterone, we also tested pregnenolone (3 μ M) with both the σ_1 antagonist, DuP734 (100 nM) and the σ_2 antagonist, Lu-28 (1 nM). Both DuP734 and Lu-28 completely reversed the inhibition by pregnenolone (Fig. 2B) to levels not different from control stimulated release.

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

To determine if PKC β is involved in the inhibition of NMDA-stimulated [3 H]dopamine release, we constructed dose-response curves for the PKC β -selective inhibitor LY379196 against both progesterone and pregnenolone. We have previously shown that LY379196 blocked (+)pentazocine mediated inhibition of NMDA-stimulated [3 H]dopamine release in a concentration-dependent manner (Nuwayhid and Werling, 2003). LY379196 has K_i values at β I and β II isozymes of 18 and 16 nM, and all other isozymes including PKC α , PKC δ , PKC γ , PKC ϵ and PKC η > 300 nM (Dr. Louis Vignati, personal communication). As seen in Figure 4A,

LY379196 completely reversed the inhibition of release by 1 μM progesterone at both 30 nM and 100 nM. At 30 nM LY379196, completely reversed the inhibition of release by 3 μM pregnenolone (Figure 4B).

Progesterone and pregnenolone are synthesized from cholesterol, as demonstrated in neural cells in culture (Guarneri et al., 2000). We therefore tested cholesterol to determine if it, as the parent compound, displayed any effects on NMDA-stimulated [^3H]dopamine release. Cholesterol (25 μM) did not affect NMDA-stimulated release of [^3H]dopamine (Figure 5).

Lastly, to verify the effects seen by pregnenolone on NMDA-stimulated [^3H]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 β -hydroxysteroid dehydrogenase, an enzyme that converts pregnenolone to progesterone ($K_i = 50$ nM; Takahashi et al., 1990). As seen in Figure 6, inhibition of [^3H]dopamine release by 3 μM pregnenolone in the presence of trilostane was not significantly different compared to the inhibition of [^3H]dopamine release by 3 μM pregnenolone alone. We also verified that progesterone was acting through σ receptors and not progesterone receptors by testing 1 μM progesterone in the presence of 10 μM mifepristone, a progesterone receptor antagonist. As seen in Figure 7, there was no difference in the inhibition seen by 1 μM progesterone alone compared with 1 μM progesterone in the presence of 10 μM mifepristone.

Discussion

The identity of the endogenous ligand for σ receptors has been equivocal. Su et al. (1988) found that progesterone, deoxycortisone and testosterone competed for [3 H]SKF10,047 at σ_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.; Guarneri, et al., 2000).

Steroids exhibit genomic and non-genomic effects (Ruppert and Holsboer, 1999). In the current study, we examined presumably non-genomic effects of steroids on NMDA-stimulated [3 H]dopamine release in rat striatal tissue via σ receptors. The effects on regulation of dopamine release occur within a relatively short time frame since 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 σ receptors they should behave similarly to prototypic ligands, such as (+)pentazocine.

We found that both progesterone and pregnenolone inhibited NMDA-stimulated [3 H]dopamine release. Both inhibited release in a concentration-dependent manner, with a maximum of about 25-30%, similar to the inhibition seen by (+)pentazocine in studies by Gonzalez-Alvear and Werling (1994; 1995). The IC_{50} of progesterone (300 nM) for inhibition of NMDA-stimulated [3 H]dopamine release was similar to its K_i value of 270 nM in competing for binding to σ receptors (Su et al., 1988). Pregnenolone showed an IC_{50} in the range of 300 nM-1 μ M. A K_i value at σ receptors in brain tissue has not been reported, but we found that pregnenolone competed for σ_1 binding with a K_i value of 980 ± 340 nM in SH-SY5Y cells (Werling, 2002). Su et al (1988) reported a K_i value of 3.2 μ M for pregnenolone sulfate binding

to σ_1 receptors. These findings support that pregnenolone acts via σ receptors to inhibit NMDA-stimulated [^3H]dopamine release.

The hypothesis that progesterone and pregnenolone act as σ agonists in our assay was confirmed by the action of σ antagonists. The inhibition of NMDA-stimulated [^3H]dopamine release by 1 μM progesterone and 3 μM pregnenolone was fully reversed by the σ_1 antagonist DuP734 ($K_i=10$ nM) (Culp et al., 1992) at 100 nM and the σ_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 σ_1 and σ_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 σ_1 or σ_2 receptors based on reversal by subtype-selective antagonists. However, in our assays examining the effects of σ_2 agonists on amphetamine-stimulated [^3H]dopamine release from rat striatal slices, we found similar antagonism of effect by σ_1 and σ_2 antagonists (Liu, et al., 2001) to that seen for the current steroid experiments. The σ_1 and σ_2 receptors are relatively small ($\sigma_1 = 28$ kDa, Hanner et. al, 1996; $\sigma_2 = 22$ kDa, Hellewell, et. al, 1990). Perhaps upon activation of σ receptor subtypes by progesterone and pregnenolone the receptors interact with each other, which in turn inhibits [^3H]dopamine release. In this situation, competing off the steroid agonist with either σ_1 or σ_2 antagonists would prevent regulation of dopamine release. Reports in which σ receptor protein was purified and detected via photoaffinity labeling (Schuster et al, 1995) or with antibody selective for σ receptor (Hanner et al., 1996), show a band with a molecular

weight of approximately 60 kDa, which could be a dimer of σ_1 and/or σ_2 receptors. Neuroactive steroids are known to modulate the actions of GABA_A and NMDA receptors (Ruppert and Holsboer, 1999). While 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, as σ receptors regulating dopamine release have been localized to that location (Gonzalez and Werling, 1995). Regardless of whether GABA_A 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 σ receptor antagonists.

The concentrations of DHEA and testosterone tested were chosen based on their reported K_i values from binding studies. While both competed for σ receptor binding (Klein and Masacchio, 1994; Su et al., 1988), our data do not indicate that the σ 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 σ antagonist activity, neither reversed the inhibition of [³H]dopamine release by progesterone or pregnenolone. DHEA and testosterone may bind to σ receptors but not have either agonist or antagonist properties in our system. It is possible that higher or lower concentrations could have produced effects, though this would not be predicted based on reported affinities at σ receptors. Further confirming that σ 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 [³H]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 3 β -hydroxysteroid

dehydrogenase enzyme that converts pregnenolone into progesterone. Therefore, both progesterone and pregnenolone appear to act as σ receptors agonists in the regulation of dopamine release.

Monnet et al. (1995) showed that DHEA S potentiated [^3H]norepinephrine release from rat hippocampal slices and this response was reversed by both σ antagonists haloperidol and BD-1063. In a later paper (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 [^3H]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 σ 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. used a concentration of NMDA four times that used by us in the current experiment and in our earlier 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 σ agonists were mediated by different populations of σ receptor subtypes depending upon the agonist used. It appears that our responses are due to activation of only σ_1 and σ_2 subtypes only as identified by selective antagonists.

Other groups have also reported effects of steroids that were mediated by σ receptors. PREG S increase spontaneous glutamate release via activation of a presynaptic $G_{i/o}$ -coupled σ receptor (Meyer et al., 2002). Since steroid sulfotransferases and sulfatases are present in the CNS (Rajkowski et al., 1997), both sulfated and non-sulfated forms could be present in the brain and modulate neurotransmitter release. However, the permeability of steroids is reduced by the

addition of sulfate; if σ 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 σ receptor mediated processes. GF109203x, a PKC inhibitor, abolished σ_2 receptor-mediated regulation of DAT activity (Derbez et al., 2002). Morin-Surun et al. (1999) showed a parallel translocation of σ receptors and PKC β I and PKC β II from the cytosol to the plasma membrane upon σ 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 PKC β 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 σ 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 PIP $_2$ hydrolysis, leading to the formation of DAG and IP $_3$, presumably due to the action of Ca^{2+} -dependent PKC (Thomas and Meizel, 1989). Estrogen increased IP $_3$ concentrations and stimulated PKC α levels in

membrane fraction of HEPG2 cells through a non-classical steroid mechanism (Marino et al., 1998). Lastly, in studies by Condliffe et al. (2001), 17- β -estradiol regulated Cl⁻ secretion in rat colonic epithelium, and regulation is blocked by the chelation of Ca²⁺ 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 β isozymes. This reinforces the possibility that steroids have non-genomic actions and can signal through PLC/PKC via their actions at σ receptors.

In conclusion, both progesterone and pregnenolone have been identified as σ receptor agonists in the modulation of NMDA-stimulated [³H]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 [³H]dopamine release mediated by progesterone and pregnenolone involves a PKC signaling system most likely to involve the PKC β isozyme. Our findings in this study further support steroids as candidates for endogenous ligands at σ receptors.

Acknowledgements

This work was supported by a grant from NIDA (DA06667) and a Faculty Enhancement Research Award from GWUMC to LLW. We thank Dr. John Puddefoot (Queen Mary and Westfield College, London) for the gift of trilostane, Dr. Rob Zaczek (DuPont Merck) for the gift of DuP734, and Dr. Connie Sanchez (H. Lundbeck) for the gift of Lu28-179. We also thank Dr. Nancy Pilotte (NIDA) for helpful suggestions regarding experimental design using steroids.

References

- Bastianetto S, Monnet F, Junien J-L and Quirion R (1999) Steroidal modulation of sigma receptor function in *Contemporary endocrinology: Neurosteroids: A New Regulatory Function on the Nervous system* (Baulieu EE, Robel P and Schumacher M eds), Humana Press, pp191-205, Totowa, NJ.
- Baulieu EE and Robel P (1996) Dehydroepiandrosterone and dehydroepiandrosterone sulfate as Neuroactive steroids. *J Endocrinol* 150 Suppl:S221-39.
- Bergeron R, de Montigny C, Debonnel G (1996) Potentiation of neuronal NMDA response induced by dehydroepiandrosterone and its suppression by progesterone: effects mediated via sigma receptors. *J Neurosci* 16:1193-1202
- Condliffe SB, Doolan CM and Harvey BJ (2001) 17- β -estradiol acutely regulates Cl⁻ secretion in rat distal colonic epithelium. *J Physiol (London)* 530:47-54.
- Culp SG, Rominger D, Tam SW and De Souza EB (1992) [³H]DuP 734 [1-(cyclopropylmethyl)-4-(2'-(4"-fluorophenyl)-2'-oxoethyl)-piperidine HBr]: a receptor binding profile of a high-affinity novel sigma receptor ligand in guinea pig brain. *J Pharmacol Exp Ther* 263(3):1175-87.
- Derbez AE, Moody RM and Werling LL (2002) Sigma₂ receptor regulation of dopamine transporter activity via protein kinase C. *J Pharmacol Exp Ther* 301:306-314.
- Falkenstein E, Tillmann H-C, Christ M, Feuring M and Wehling M (2000) Multiple actions of steroid hormones-a focus on rapid, nongenomic effects. *Pharm Rev* 52:513-555.
- Gibbs T and Farb D (2000) Dueling enigmas: neurosteroids and sigma receptors in the limelight. *Science's stke* November 28 (60)PE1.

Gonzalez-Alvear GM and Werling LL (1994) Regulation of [³H]dopamine release from rat striatal slices by sigma receptor ligands. *J Pharmacol Exp Ther* 271:212-219.

Gonzalez-Alvear GM and Werling LL (1995) Sigma₁ receptors in rat striatum regulate NMDA stimulated [³H]dopamine release via a presynaptic mechanism. *Eur J Pharmacol* 294:713-719.

Guarneri P, Cascio C, Piccoli T, Piccoli F and Guarneri R (2000) Human neuroblastoma SH-SY5Y cell line: neurosteroid-producing cell line relying on cytoskeletal organization. *J Neurosci Res* 60:656-665.

Hellewell SB, Bowen WD (1990) A sigma-like binding site in rat pheochromocytoma (PC12): decreased affinity for (+)benzomorphans and lower molecular weight suggest a different σ receptor from that of guinea pig brain. *Brain Res* 527:244-253.

Hanner M, Moebius FF, Flandorfer A, Knaus HG, Striessnig J, Kemper E and Glossman H (1996) Purification, molecular cloning and expression of the mammalian sigma₁ binding site. *Proc Natl Acad Sci (USA)* 93:8072-8077.

Hu ZY, Bourreau E, Jung-Testas I, Robel P and Baulieu EE (1987) Neurosteroids: oligodendrocyte mitochondria convert cholesterol to pregnenolone. *Proc Natl Acad Sci (USA)* 84:8215-8219.

Izenwasser S, Thompson-Montgomery DT, Deben SE, Chowdhury IN and Werling LL (1998) Modulation of amphetamine-stimulated (transporter-mediated) dopamine release by sigma₂ receptor agonists and antagonists in vitro. *Eur J Pharmacol* 346:189-196.

Jung-Testas Z, Hu ZY, Baulieu EE and Robel P (1989) Neurosteroids: biosynthesis of pregnenolone and progesterone in primary cultures of rat glial cells. *Endocrinology* 125:2083-2091.

- Klein M and Musacchio JM (1994) Effects of cytochrome P-450 ligands on the binding of [³H]dextromethorphan and sigma ligands on to guinea pig brain in *Sigma Receptors* (Itzhak Y. ed), Academic Press, pp 243-262, San Diego, CA.
- Liu X, Nuwayhid S, Christie M, Kassiou M and Werling LL (2001) Trishmocabanes: novel σ ligands modulate amphetamine-stimulated [³H]dopamine release. *Eur J Pharmacol* 422: 39-45.
- Marino PJ, Pallottini V and Trentalance A (1998) Estrogens cause rapid activation of IP3-PKC signal transduction in HEPG2 cells. *Biochem Biophys Res Commun* 245:254-258.
- Martin WR, Eades CG, Thompson JA, Huppler RE and Gilbert PE (1976) The effects of morphine- and nalorphine-type drugs in the non-dependent and morphine-dependent chronic spinal dog. *J Pharm Exp Ther* 197:517-532.
- Maurice T, Phan VL, Urani A, Kamei H, Noda Y, Nabeshima T (1999) Neuroactive steroids as endogenous effectors for the sigma1 (σ_1) receptor: pharmacological evidence and therapeutic opportunities. *Jpn J Pharmacol* 81:125-155.
- Maurice T, Phan VL, Urani A and Guillemain (2001) Differential involvement of the sigma-1 receptor in the anti-amnesic effect of neuroactive steroids as demonstrated using an antisense oligonucleotide strategy in the mouse. *Br J Pharmacol* 134:1731-1741.
- McCann DM and Su TP (1994) Sigma₁ and sigma₂ sites in rat brain: comparison of regional, ontogenetic, and subcellular patterns. *Synapse* 17:182-189.
- Meyer DA, Carta M, Partridge D, Covey DF and Valenzuela CF (2002) Neurosteroids enhance spontaneous glutamate release in hippocampal neurons: possible role of metabotropic σ_1 like receptors. *J Biol Chem* 277(2):28725-28732.
- Moltzen EK, Perregard TA and Meier E (1995) σ ligands with subnanomolar affinity and

- preference for the σ_2 binding site 2. spiro-joined benzofuran, isobenzofuran and benzopyran piperidines. *J Med Chem* 38:2009-2017.
- Monnet FP, Mahe V, Robel P and Baulieu EE (1995) Neurosteroids, via sigma receptors, modulate the [3 H]norepinephrine release evoked by NMDA in the rat hippocampus. *Proc Natl Sci (USA)* 92:3774-3778.
- Morin-Surin MP, Collin T, Denavit-Saubie M, Baulieu EE and Monnet FP (1999) Intracellular sigma $_1$ receptor modulates phospholipase C and protein kinase C activities in the brainstem. *Proc Natl Sci (USA)* 96:8196-8199.
- Nuwayhid S and Werling LL (2003) Sigma $_1$ receptor agonist-mediated regulation of NMDA-stimulated [3 H]dopamine release is dependent upon protein kinase C. *J Pharm Exp Ther* 304(1):364-369.
- Rajkowski KM, Robel P and Baulieu EE (1997) Hydroxysteroid sulfotransferase activity in the rat brain and liver as a function of age and sex. *Steroids* 62(5):427-436.
- Ruppert R and Holsboer F (1999) Neuroactive steroids: mechanisms of action and neuropsychopharmacological perspectives. *Trends Neurosci* 22:410-416.
- Schuster DI, Arnold FJ and Murphy RB (1995) Purification, pharmacological characterization and photoaffinity labeling of sigma receptors from rat and bovine brain. *Brain Res* 670:14-28.
- Snell LD, Iorio KR, Tbakoff B and Hoffman PL (1994) Protein kinase C activation attenuates NMDA-induced increases in intracellular calcium in cerebellar granule cells. *J Neurochem* 62:1783-1789.
- Su TP, London ED and Jaffe JH (1988) Steroid binding at sigma receptors suggest a link between endocrine, nervous and immune systems. *Science* 240:219-221.

- Swartz S, Pohl P and Zhou GZ (1989) Steroid binding at σ - "opioid" receptors. *Science* 246: 1635-1637.
- Takahashi M, Luu-The V, Labrie F (1990) Inhibitory effect of synthetic progestins, 4-MA and cyanoketone on human placental 3β -hydroxysteroid dehydrogenase/5—4-ene isomerase activity. *J Steroid Biochem Mol Biol* 37:231-236.
- Thomas P and Meizel S (1989) Phosphatidylinositol 4,5 biphosphate hydrolysis in human sperm stimulated with follicular fluid or progesterone is dependent on upon Ca^{2+} influx. *J Biochem* 264:539-546.
- Vilner BJ and Bowen WD (2000) Modulation of cellular calcium by sigma-2 receptors: release from intracellular stores in human SK-N-SH neuroblastoma cells. *J Pharm Exp Ther* 292:900-911.
- Werling L.L. (2002) Sigma receptor-active steroids and neurotransmitter release *Int J Psychopharmacol* 5, S28.
- Werling LL, Frattali A, Portoghese PS, Takemori AE and Cox BM (1988). Kappa receptor regulation of dopamine release from striatum and cortex of rat and guinea pig. *J Pharm Exp Ther* 246: 282-286 (1988).

Footnotes

*This work was supported by a grant from NIDA and a Faculty Research Enhancement Fund award to LLW.

Reprint requests and correspondence should be addressed to:

Linda L. Werling, Ph.D.

Department of Pharmacology

The George Washington University

2300 Eye Street, Northwest

Washington, DC 20037

Fig. 1. Lack of effect of 10 μ M DHEA, 3 or 10 μ M testosterone on NMDA-stimulated [3 H]dopamine release. Release of [3 H]dopamine from slices of rat striatal tissue was stimulated by 25 μ M NMDA or 25 μ M NMDA in the presence of DHEA or testosterone as indicated. Statistical analysis by ANOVA, performed on untransformed data (ratios of S2/S1) indicated no significant difference in 25 μ M NMDA alone compared with 25 μ M NMDA in the presence of DHEA or testosterone. Data are expressed as % control NMDA-stimulated release. N = 3 independent experiments in which each treatment was tested in triplicate. Note break in Y-axis.

Fig. 2. Concentration-response curves for (A) progesterone and (B) pregnenolone on NMDA-stimulated [³H]dopamine release, and reversal by σ receptor antagonists. Release of [³H]dopamine from slices of rat striatal tissue was stimulated by 25 μ M NMDA alone or 25 μ M NMDA in the presence of progesterone or pregnenolone as indicated, with or without the σ_1 antagonist DuP734 (100 nM) or the σ_2 antagonist Lu28-179 (1 nM). Data are expressed as % control NMDA-stimulated release. *Significantly different from NMDA alone at $p < 0.05$. # Significantly different from NMDA in the presence of progesterone or pregnenolone as indicated, $p < 0.05$. $N \geq 3$ independent experiments in which each treatment was tested in triplicate. Note breaks in Y-axes.

Fig. 3. Lack of effect of testosterone or DHEA on the inhibition of NMDA-stimulated [³H]dopamine release by (A) 1 μM progesterone and (B) 3 μM pregnenolone. Release of [³H]dopamine from slices of rat striatal tissue was stimulated by 25 μM NMDA alone or 25 μM NMDA in the presence of agonist steroid, as indicated, with or without potential antagonist steroid testosterone (3 μM) or DHEA (10 μM). Data are expressed as % 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.

Fig. 4. Reversal of steroid-mediated inhibition of NMDA-stimulated [³H]dopamine release by the PKC β -selective inhibitor LY379196. Release of [³H]dopamine from rat striatal slices was stimulated by 25 μ M NMDA alone or 25 μ M NMDA in the presence of (A) 1 μ M progesterone or (B) 3 μ M pregnenolone, as indicated, with or without the indicated concentration of LY379196. Data are expressed as % control NMDA-stimulated release. *Significantly different from NMDA alone at $p < 0.05$, $N = 3$ independent experiments. # Significantly different from 1 μ M progesterone or 3 μ M 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.

Fig. 5. Lack of effect of cholesterol on NMDA-stimulated [³H]dopamine release. Release of [³H]dopamine from slices of rat striatal tissue was stimulated by 25 μM NMDA alone or 25 μM NMDA in the presence 25 μM cholesterol, as indicated. There was no significant difference in release of [³H]dopamine stimulated by 25 μM NMDA alone compared to 25 μM NMDA in the presence of 25 μM cholesterol. Data are expressed as % control NMDA-stimulated release. N=3 independent experiments in which each treatment was tested in triplicate. Note break in Y-axis.

Fig. 6. Lack of significant effect of trilostane on pregnenolone-mediated inhibition of NMDA-stimulated [³H]dopamine. Release of [³H]dopamine from slices of rat striatal tissue was stimulated by 25 μM NMDA alone or 25 μM NMDA in the presence 3 μM pregnenolone, as indicated, with or without 25 μM trilostane. Data are expressed as % control NMDA-stimulated release. *Significantly different from NMDA alone at $p < 0.05$, $N = 3$ independent experiments in which each treatment was tested in triplicate. Note break in Y-axis.

Fig. 7. Lack of effect of mifepristone on the inhibition of NMDA-stimulated [³H]dopamine release by 1 μM progesterone. Release of [³H]dopamine from slices of rat striatal tissue was stimulated by 25 μM NMDA alone or 25 μM NMDA in the presence 1 μM pregnenolone, as indicated, with or without mifepristone. Data are expressed as % control NMDA-stimulated release. *Significantly different from NMDA alone at $p < 0.05$, $N = 3$ independent experiments in which each treatment was tested in triplicate. Note break in Y-axis.

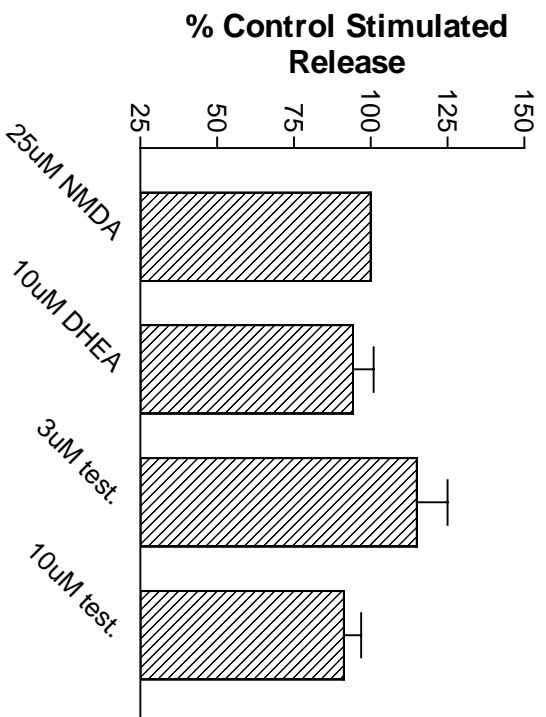


Figure 1

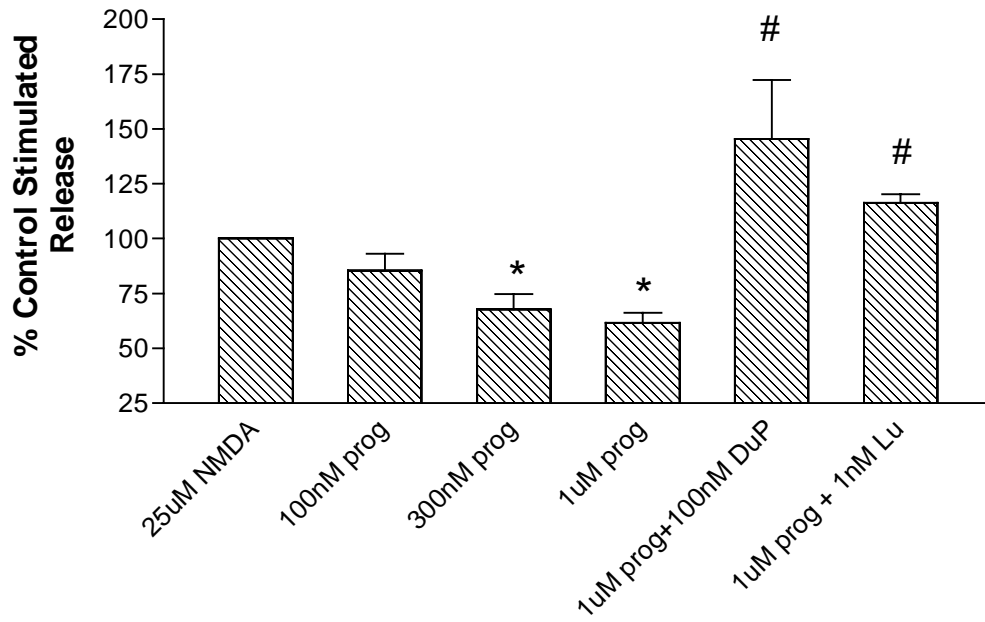


Figure 2A

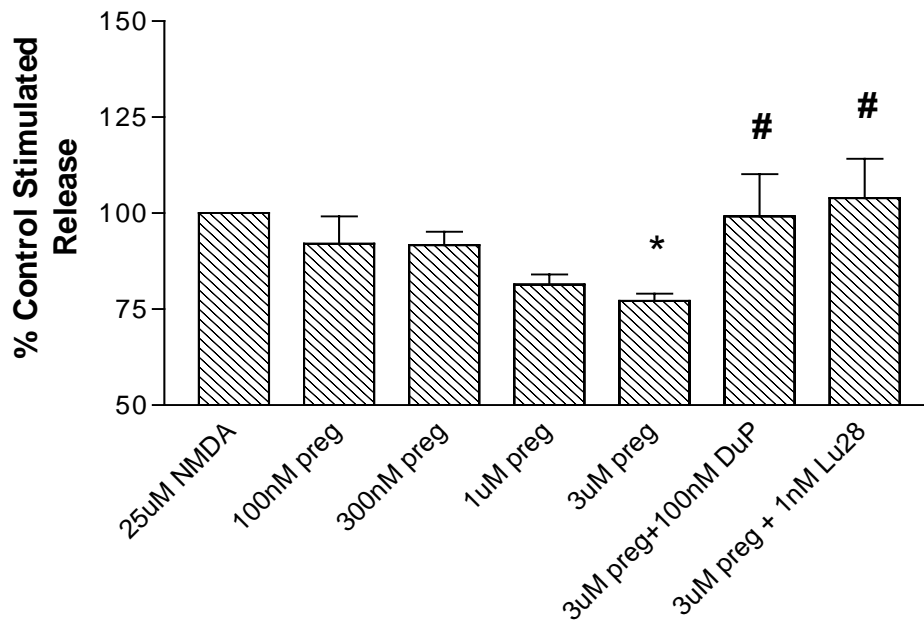


Figure 2B

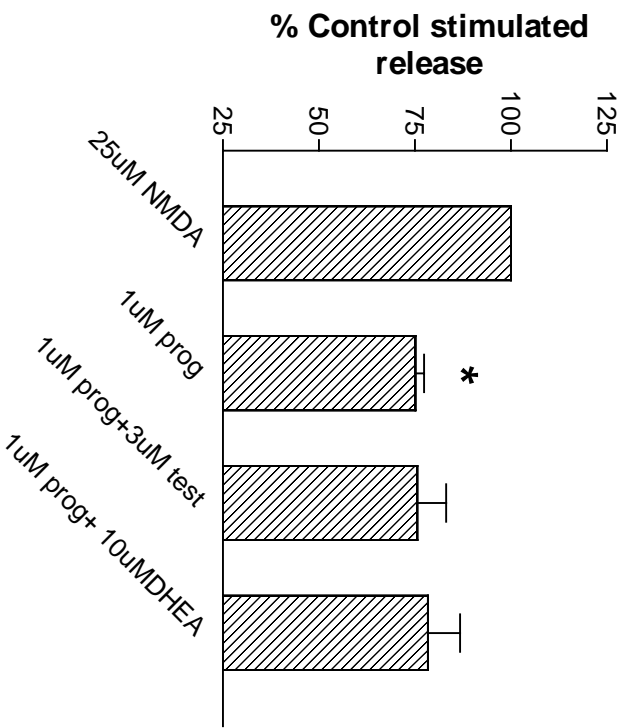


Figure 3A

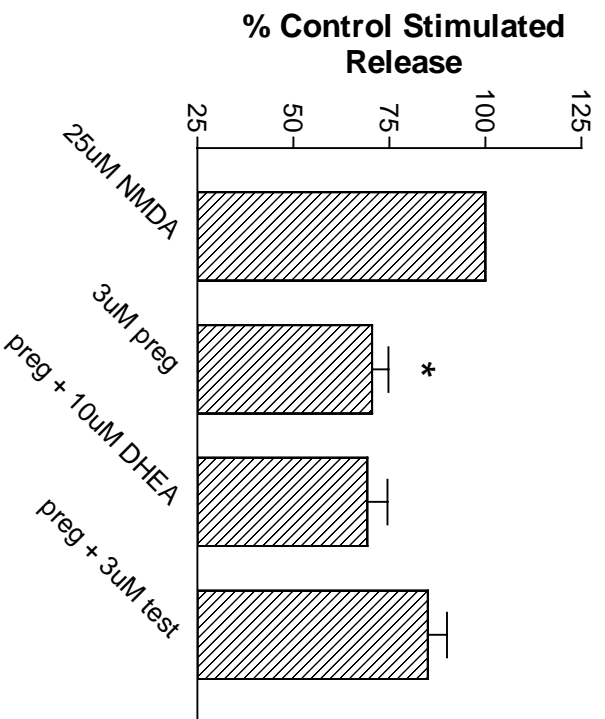


Figure 3B

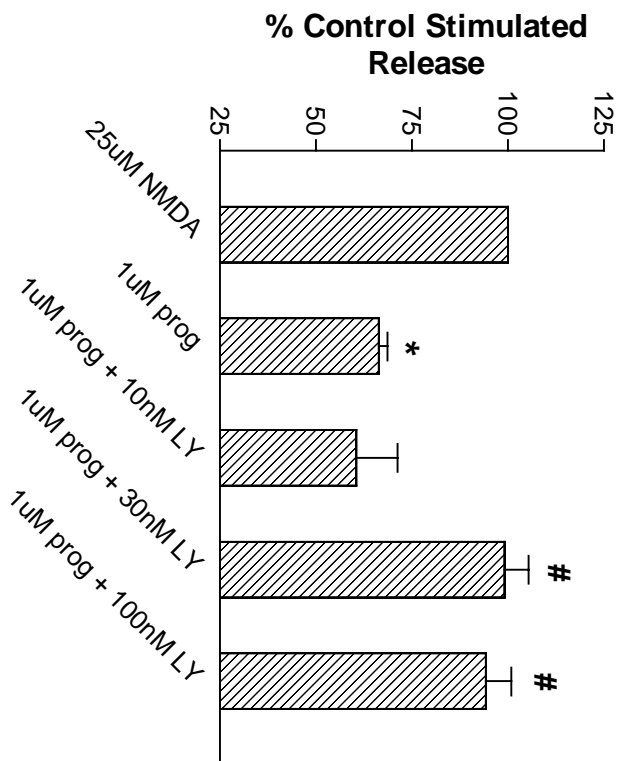


Figure 4A

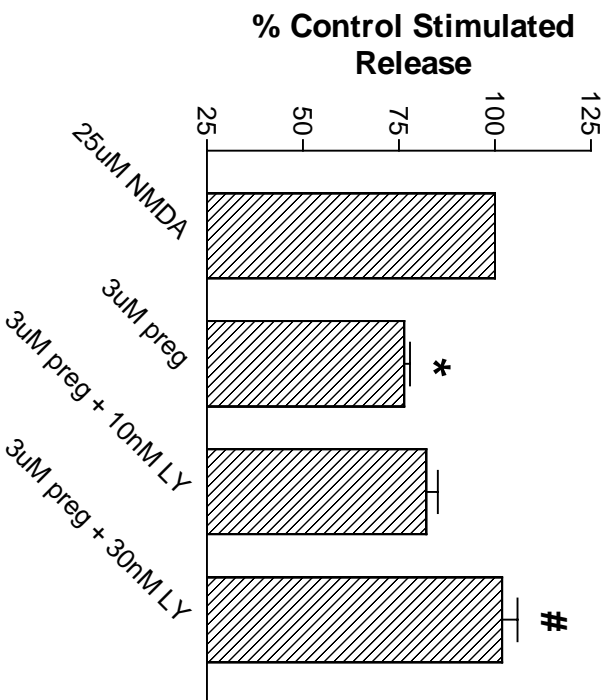


Figure 4B

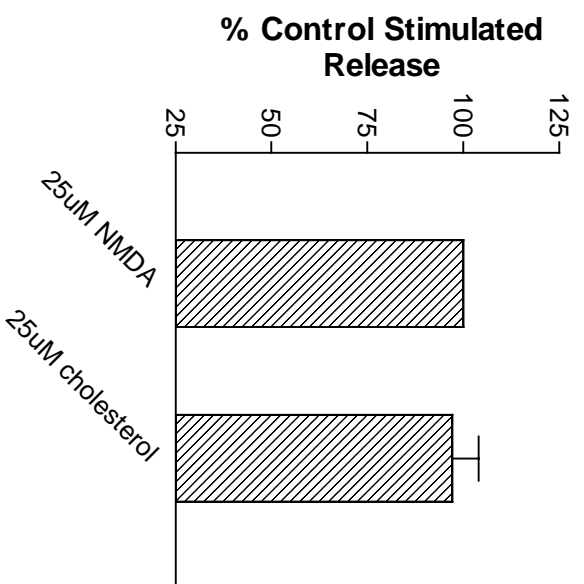


Figure 5

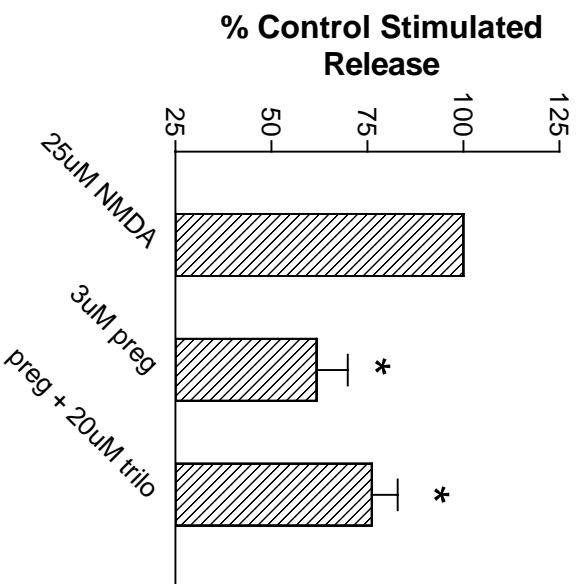


Figure 6

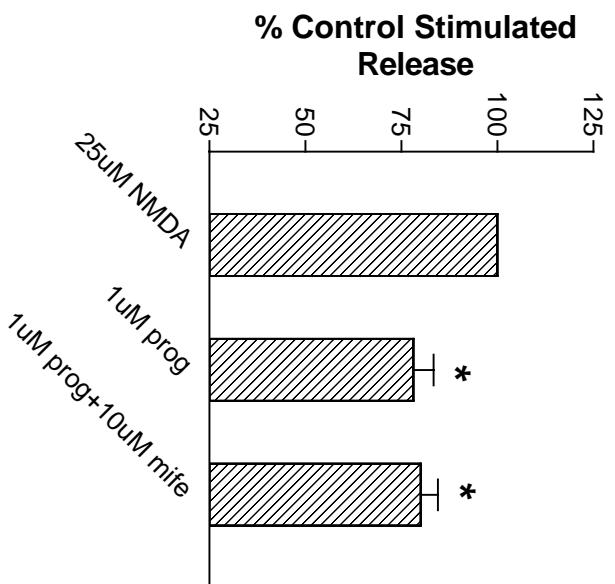


Figure 7