NIH Public Access
Author Manuscript
*J Neurochem*. Author manuscript; available in PMC 2009 September 25.

Published in final edited form as:
*J Neurochem*. 2008 October ; 107(2): 510–521. doi:10.1111/j.1471-4159.2008.05627.x.

# Pregnenolone sulfate induces NMDA receptor dependent release of dopamine from synaptic terminals in the striatum

**Matthew T. Whittaker**, **Terrell T. Gibbs**, and **David H. Farb**
Laboratory of Molecular Neurobiology, Department of Pharmacology & Experimental Therapeutics, Boston University School of Medicine, Boston, Massachusetts, USA

## Abstract

Neuromodulators that alter the balance between lower-frequency glutamate-mediated excitatory and higher-frequency GABA-mediated inhibitory synaptic transmission are likely to participate in core mechanisms for CNS function and may contribute to the pathophysiology of neurological disorders such as schizophrenia and Alzheimer's disease. Pregnenolone sulfate (PS) modulates both ionotropic glutamate and $GABA_A$ receptor mediated synaptic transmission. The enzymes necessary for PS synthesis and degradation are found in brain tissue of several species including human and rat, and up to 5 nM PS has been detected in extracts of postmortem human brain. Here, we ask whether PS could modulate transmitter release from nerve terminals located in the striatum. Superfusion of a preparation of striatal nerve terminals comprised of mixed synaptosomes and synaptoneurosomes with brief-duration (2 min) pulses of 25 nM PS demonstrates that PS increases the release of newly accumulated [$^3$H]dopamine ([$^3$H]DA), but not [$^{14}$C]glutamate or [$^3$H]GABA, whereas pregnenolone is without effect. PS does not affect dopamine transporter (DAT) mediated uptake of [$^3$H]DA, demonstrating that it specifically affects the transmitter release mechanism. The PS-induced [$^3$H]DA release occurs via an NMDA receptor (NMDAR) dependent mechanism as it is blocked by D-2-amino-5-phosphonovaleric acid. PS modulates DA release with very high potency, significantly increasing [$^3$H]DA release at PS concentrations as low as 25 pM. This first report of a selective direct enhancement of synaptosomal dopamine release by PS at picomolar concentrations via an NMDAR dependent mechanism raises the possibility that dopaminergic axon terminals may be a site of action for this neurosteroid.

### Keywords

neuroactive steroid; NMDA receptors; striatum; superfusion; synaptoneurosome; synaptosome

Pregnenolone sulfate (PS) is a neuroactive steroid that directly modulates glutamate and $GABA_A$ receptor function, suggesting that it may regulate the balance between excitatory and inhibitory neurotransmission (Farb and Gibbs 1996; Gibbs and Farb 2000). PS also modulates the activity of AMPA and kainate receptors (Wu *et al.* 1991), σ receptors (Monnet *et al.* 1995; Hayashi *et al.* 2000), and certain voltage-gated calcium channels (Bukusoglu and Sarlak 1996; Hige *et al.* 2006).

Modulation of synaptic transmission by PS has been demonstrated in multiple experimental paradigms. PS potentiates spontaneously occurring excitatory postsynaptic currents (EPSCs) in hippocampal cell cultures (Park-Chung *et al.* 1997; Meyer *et al.* 2002) and in slices prepared from rat pre-limbic cortex (Dong *et al.* 2005), as well as evoked EPSCs from hippocampal

Address correspondence and reprint requests to David H. Farb, Laboratory of Molecular Neurobiology, Department of Pharmacology & Experimental Therapeutics, Boston University School of Medicine; 715 Albany Street; Boston, MA 02118, USA. dfarb@acs.bu.edu.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

(Schiess *et al.* 2006) and calyx of Held synapses (Hige *et al.* 2006). PS also augments NMDAR independent long term potentiation in the rat hippocampus via modulation of L-type $Ca^{2+}$ channels and σ receptors (Sabeti *et al.* 2007), and a PS-like retrograde modulatory factor plays a role in plasticity of immature hippocampal synapses (Mameli *et al.* 2005).

Enzymes for synthesis of pregnenolone from cholesterol (cytochrome P450 scc) and sulfation of pregnenolone to PS (neurosteroid sulfotransferase ST2A1, SULT2B1a) are present in neural tissue (Hojo *et al.* 2004; Kohjitani *et al.* 2006). Pregnenolone is inactive at both glutamate and $GABA_A$ receptors, indicating that the negatively charged sulfate group of PS is essential for its modulatory activity. Whereas pregnenolone is neutral and lipophilic, permitting rapid permeation across cell membranes, sulfation to form PS results in a negatively charged steroid that could be compartmentalized intracellularly. Pregnenolone sulfotransferase is present in rat C6 glioma cells, where its activity is regulated by AMPA receptors (Kohjitani *et al.* 2008). Steroid sulfatases are present in rodent, bovine, monkey, and human brain and offer a potential mechanism for inactivation of PS (Compagnone *et al.* 1997; Mellon *et al.* 2001; Plassart-Schiess and Baulieu 2001). Collectively, these observations indicate that PS satisfies several classical criteria for identification as a neurotransmitter or neuromodulator: it is synthesized in nervous tissue, has specific receptor pharmacology, and is inactivated by removal of the sulfate group, but the role of PS within the nervous system remains unresolved.

Whether PS is stored and released at physiologically active concentrations by either neurons or glia has remained a controversial issue. The average tissue level of PS in extracts of postmortem aged human brain was found to be 2.8 nM in frontal cortex and 4.6 nM in cerebellum, while blood plasma contains about 380 nM free sulfated steroid. In rat, however, the average level of PS in adrenal tissue was 14 nM but was only 0.64 nM in two of five samples from anterior brain (limit of detection: 0.38 nM) and undetectable in other brain regions (Liere *et al.* 2004). Another study (Ebner *et al.* 2006) failed to detect PS in extracts of pooled whole rat brain, with a reported detection limit of 141 pM. Nevertheless, the inability to detect gross tissue levels of PS in various regions of rat brain does not exclude the possibility that significant amounts of this steroid might be present on a cellular level (Schumacher *et al.* 2008).

Infusion of 10 nM PS into rat striatum via reverse microdialysis results in a significant increase in dopamine (DA) overflow in the striatum that involves NMDA receptor (NMDAR) function, but not σ receptor function (Sadri-Vakili *et al.* 2008; Gibbs *et al.* 2006). To ask whether this high affinity effect of PS was exerted via modulation of presynaptic NMDARs located on dopaminergic terminals, we investigated the effect of PS on DA release *ex vivo* from a preparation of striatal nerve terminals comprised of mixed synaptosomes and synaptoneurosomes (SSNs). We report that PS at concentrations as low as 25 pM induces [$^3$H]DA release from striatal SSNs, while pregnenolone is without effect, suggesting that subnanomolar concentrations of PS could modulate nigro-striatal DA release. To our knowledge, this study is the first to report direct neurosteroid enhancement of NMDAR-dependent synaptosomal transmitter release at subnanomolar concentrations of steroid.

## Materials and methods

### Subjects

Male Sprague-Dawley rats (225-300 g) from Charles River Laboratories (Wilmington, MA, USA) were housed in shoe-box cages (two rats/cage) and provided with food and water *ad libitum*. The cages were kept in a temperature-controlled room with a 12-h light/dark cycle. All experiments were performed during the light cycle. All procedures were carried out under a protocol approved by the Boston University School of Medicine IACUC.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript



## Materials

[7,8-$^3$H]dopamine ([$^3$H]DA: 45 Ci/mmol), L-[U-$^{14}$C]glutamate ([$^{14}$C]glutamate: 253 mCi/mmol), and 4-amino-*n*-[2,3-$^3$H]butyric acid ([$^3$H]GABA: 94 Ci/mmol) were from Amersham (Buckinghamshire, UK). Steroids were purchased from Steraloids (Newport, RI, USA) and prepared as 500× stock solutions in dimethyl sulfoxide (DMSO). The final DMSO concentration of all perfusion solutions in experiments involving steroid application was 0.2%. Chemicals used in preparation of tissue for electron microscopy were purchased from Electron Microscopy Sciences (Hatfield, PA, USA). All other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

## Synaptosome/synaptoneurosome preparation

Synaptosome/synaptoneurosomes (SSNs) were prepared according to the methods of (Pittaluga *et al.* 2001) with minor modifications. Rats were killed by decapitation and brains removed immediately. Striatal tissue was dissected out and placed in 12 mL of ice cold homogenization buffer (320 mM sucrose, 4 mM sodium phosphate buffer, pH 7.4). Tissue was homogenized by 12 strokes in a motor driven glass-Teflon homogenizer at 4°C, and centrifuged for 10 min at 1000 *g*. The supernatant (S1) was removed and centrifuged at 12 000 *g* for 20 min. The resulting pellet (P2) was then resuspended in 12 mL ice cold homogenization buffer and re-centrifuged for 20 min at 12 000 *g* to obtain the final synaptosome/synaptoneurosome (SSN) pellet. The preparation was maintained on ice in pellet form until immediately prior to experimentation.

## Percoll purified SSNs

Percoll purified SSNs were prepared according to the methods of (Dunkley *et al.* 1988) with minor modifications. The resuspended P2 pellet was applied to a discontinuous Percoll gradient and centrifuged at 32 500 *g* for exactly 5 min in a Beckman XL-90 ultracentrifuge. The band containing SSNs at the 23%/10% interface was removed and placed in ice-cold $Mg^{2+}$-free artificial cerebrospinal fluid (aCSF; 125 mM NaCl, 3 mM KCl, 1.2 mM $CaCl_2$, 1 mM $NaH_2PO_4$, 22 mM $NaHCO_3$, 10 mM glucose; aerated with 95% $O_2$/5% $CO_2$; pH 7.2–7.4). The SSNs were pelleted by centrifugation at 27 000 *g* and resuspended in aCSF. The SSNs were centrifuged again at 27 000 *g* and subsequently maintained on ice in pellet form until experimentation.

## Electron microscopy

Percoll-purified striatal SSNs were fixed in 4% paraformaldehyde plus 0.1% glutaraldehyde in aCSF for 1 h at 4°C. The SSNs were then pelleted by centrifugation at 2000 *g* for 2 min in a benchtop centrifuge (Shelton Scientific, model VSMC-13) and exposed to 3 × 5 min washes in ice cold aCSF. The pellet was resuspended for each wash step. Next, the SSN pellet was incubated with 4% osmium tetroxide/1.5% potassium ferrocyanide for 2 h at 4°C. The pellet was then dehydrated through an increasing ethanol series, washed 3 × 10 min with propylene oxide, covered in Epon-Araldite embedding mixture and left overnight at RT. The embedding mixture was replaced and the pellet was then incubated for 48 h at 60°C. 90–100 nm sections of the embedded SSN pellets were cut using an ultramicrotome (Ultratome Nova LKB2188). Sections were mounted on copper grids and counter-stained with 1% uranyl acetate in 100% methanol and viewed with a transmission electron microscope (Jeol 1200) at 60 keV.

## Radiolabeled neurotransmitter uptake experiments

The SSN pellet was resuspended in $Mg^{2+}$-free aCSF immediately prior to experimentation, divided into equal volumes, and maintained on ice. SSNs were incubated for 15 min (unless otherwise specified) with [$^3$H]DA (20 nM final concentration), [$^3$H]GABA (20 nM final concentration), or [$^{14}$C]glutamate (2.9 μM final concentration) at 37°C or 0°C in the presence

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

or absence of test compounds. SSNs were pre-incubated for 5 min at 37°C prior to initiation of uptake in time course experiments. Uptake was stopped by the addition of ice-cold aCSF, and SSNs were passed through Whatman GF/B filters in a vacuum filtration manifold (Hoefer Scientific Instruments, San Francisco, CA, USA). Filters were washed twice with aCSF and subsequently placed in scintillation vials. Ecolite (+) scintillation cocktail (MP Biomedical, Costa Mesa, CA, USA) was added, and vials were thoroughly vortexed. Vials were maintained in the dark for at least 12 h, and radioactivity remaining on filters was determined by liquid scintillation counting (Beckman model LS6000SC, Fullerton, CA, USA). Specific uptake was calculated as uptake observed at 37°C minus the uptake at 0°C. All uptake experiments were completed within 4 h of harvesting the rat brain.

### Radiolabeled neurotransmitter release experiments

The SSN pellet was resuspended in $Mg^{2+}$-free aCSF plus 0.2% DMSO and loaded by incubation with 20 nM [$^{3}$H]DA, 20 nM [$^{3}$H]GABA, or 2.9 µM [$^{14}$C]glutamate for 15 min at 37°C. In dual-label experiments, [$^{3}$H]GABA was loaded together with [$^{14}$C]glutamate. Specific uptake was confirmed for each experiment in a parallel assay that compared the uptake at 37°C to that at 0°C. Equal volumes of the loaded SSNs were then introduced between two Whatman GF/B filters (8 mm diameter) in each of six parallel superfusion chambers (three control and three experimental replicates) in a Brandel suprafusion apparatus (Model SF-06, Brandel, Gaithersburg, MD, USA). SSNs were perfused with continuously oxygenated $Mg^{2+}$-free aCSF plus 0.2% DMSO at a rate of 0.6 mL/min at 37°C. SSNs were calculated to be present as a single 20–40 µm thick layer under these experimental conditions. 0.2% DMSO had no effect on the uptake or spontaneous release of any of the neurotransmitters examined. 100 µM pargyline was included in the perfusion buffer in experiments that examined [$^{3}$H]DA release. For some experiments, 500 µM *n*-acetylcysteine (NAC) was added to the perfusion buffer to reduce spontaneous efflux of [$^{3}$H]DA from SSNs. The presence of NAC did not influence the effects of steroid application. After a 15 min initial washout period, 1-min fractions were collected from t = 15 to 35 min. Drug treatments were introduced in a 2 min pulse from t = 20 to 22 min (S1 pulse). A 2 min pulse of 500 µM nicotine or 13 mM KCl (10 mM NaCl replaced with 10 mM KCl) was applied starting at t = 31 min (nicotine) or t = 32 min (KCl) as a positive control in each experiment (S2 pulse). Drug application required the movement of a manifold from a reservoir containing bulk buffer solution to individual tubes containing drug solution, which were aligned in parallel, a process taking ~ 3 s. The direction of buffer flow was reversed during movement of the manifold to prevent air from entering the tubing, and to maintain a constant flow of buffer over the SSNs. This process resulted in a small, transient increase in transmitter release at the time of switching. The dead volume of the tubing in the superfusion apparatus is equivalent to four fractions. Consistent with this, the switching artifact effect is observed four fractions before the first fraction in which SSNs are exposed to drug in both pulse 1 (seen at t = 17 min) and pulse 2 (seen at t = 29 min).

For experiments that tested the effect of PS in the presence of antagonists, the antagonists were included in the perfusion buffer throughout the entire perfusion period. At the conclusion of each experiment, filters containing immobilized SSNs were collected and lysed with 0.6 mL 0.1 M perchloric acid. The radioactivity present in the perfused SSNs retained on the filters, and in perfusate fractions collected, was determined by liquid scintillation counting as in the uptake experiments described above. Experimental trials that did not meet predetermined criteria pertaining to a stable baseline level of DA release and response to the positive control pulse were excluded. All experiments were completed within 4 h of harvesting the rat brain.

### Statistical analysis

The amount of radiolabeled neurotransmitter released during each 1-min collection interval was expressed as a percentage of the total radioactivity present in the SSNs at the time of

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript



fraction collection, referred to as 'fractional release' (Sanz *et al.* 2000). Total radioactivity was computed as the sum of released radioactivity present in all fractions prior to the sample of interest, plus the radioactivity remaining in the SSNs at the conclusion of the experiment.

### S1 evoked neurotransmitter release

Evoked release was calculated as percentage increase over baseline. Baseline was defined as the mean fractional release value for three fractions prior to drug application. Evoked release values obtained from parallel controls were subtracted from drug evoked release values to obtain the net effect of drug application. The total effect of drug application was calculated as the sum of the net percent increase values for t = 20–22 min. These points corresponded to the points of maximal drug effect. The samples to be statistically tested for a change in transmitter release were identified based upon experimental measurement of the delay resulting from the dead volume of the apparatus combined with the perfusion rate. Depending upon the precise timing of perfusion, some of the stimulated release may be collected in the fraction immediately preceding the transmitter/drug pulse because of a small amount of variation in flow rate as compared with fraction collecting (see Fig. 2b, fraction 19).

### S2 evoked neurotransmitter release

Evoked release was calculated as percentage increase over baseline as in the calculation of the S1 effect. Baseline was defined as the mean fractional release value for t = 28–30 min. The total effect of drug application was calculated as the sum of the percent increase values for t = 31–34 min for nicotine and t = 32–35 min for KCl. These points corresponded to the points of maximal drug effect.

## Results

We examined whether PS could directly release DA from a superfused rat striatal synaptosome/synaptoneurosome mixture (SSN) that was pre-loaded with [$^3$H]DA. Two independent SSN preparations were examined by electron microscopy and found to contain numerous intact, sealed synaptic terminals that contained mitochondria, clear synaptic vesicles, and attached postsynaptic elements (Fig. 1). All of the structures in 13 fields containing synaptic contacts were characterized, and axon terminals were found to range in size from 0.2 μm–1.5 μm in diameter. The synaptic terminals observed in the SSN preparation were comprised of synaptosomes (40.3% of total synaptic junctions), characterized by an intact presynaptic terminal, synaptic junction, and ruptured postsynaptic element; and synaptoneurosomes (59.7% of total synaptic junctions), which are characterized by intact pre- and postsynaptic elements. The majority of synapses were symmetric (62.3%), indicative of inhibitory synaptic contacts.

The kinetics for specific [$^3$H]DA uptake into SSNs (Fig. 1d) provide evidence that [$^3$H]DA is taken up into axon terminals via a single component transport system. Specific [$^3$H]DA uptake increased with increasing SSN protein concentration and was blocked by the DA transport inhibitor nomifensine (Fig. 1e). Depolarization with both 100 mM KCl and 100 μM NMDA inhibits [$^3$H]DA uptake by SSNs (Fig. 1f). As expected, the degree of uptake inhibition with a very high concentration of KCl (100 mM) is much greater than that with a subsaturating concentration of NMDA (100 μM). 100 mM KCl depolarizes all terminals in the SSN preparation to approximately −12 mV (based on the Nernst equation). Depolarization to this degree is expected to release nearly all releasable DA present in terminals, and prevent any uptake of [$^3$H]DA present in the extracellular solution. 100 μM NMDA is a subsaturating concentration of NMDA, and, furthermore, NMDARs may not be present at sufficient density on DA terminals to evoke DA release (and thus inhibit uptake) to the same degree as that seen with depolarization by KCl. Finally, specific uptake of [$^3$H]DA is two-fold greater in striatal

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

SSNs as compared with whole brain SSNs (Fig. 1g). Specific uptake of [$^3$H]GABA into striatal SSNs is blocked by the GAT1 inhibitor nipecotic acid and specific [$^{14}$C]glutamate uptake is reduced by the broad spectrum EAAT inhibitor DL-TBOA (Table 1).

Superfusion studies were carried out in $Mg^{2+}$-free buffer to assess the effects of application of exogenous drug compounds on the release of radiolabeled neurotransmitter from pre-loaded striatal SSNs. A 2 min application of 300 μM NMDA + 1 μM glycine or 20 μM L-glutamate + 1 μM glycine to perfused striatal SSNs increases [$^3$H]DA release by > 70% relative to control (Fig. 2a and b), in agreement with previous reports (Johnson and Jeng 1991; Krebs *et al.* 1991; Wang 1991; Pittaluga *et al.* 2001). DL-2-amino-5-phosphonovaleric acid (D-AP5), an NMDAR competitive antagonist, blocks the ability of NMDA to stimulate [$^3$H]DA release, and partially inhibits the effect of L-glutamate (Fig. 2c). 300 μM NMDA + 1 μM glycine application also increases [$^{14}$C]glutamate and [$^3$H]GABA release from striatal SSNs (Fig. 3, Table 2). A 2 min application of 10 mM KCl also induces substantial release of [$^3$H]DA, [$^{14}$C]glutamate, and [$^3$H]GABA, while 500 μM nicotine stimulates [$^3$H]DA release to a greater degree than [$^{14}$C]glutamate or [$^3$H]GABA (Table 2).

These observations indicate the involvement of NMDA receptors in [$^3$H]DA release from nigro-striatal axon terminals. Subsequent experiments examined whether the neuroactive steroid PS could augment [$^3$H]DA release from striatal SSNs at physiologically relevant concentrations. A 2 min application of 25 nM PS significantly enhances spontaneous [$^3$H]DA release (Fig. 4a), while 25 nM pregnenolone is without effect (Fig. 4b). The magnitude of the [$^3$H]DA release induced by PS is 44% of the [$^3$H]DA release induced by application of 300 μM NMDA + 1 μM glycine (Table 2). PS-induced [$^3$H]DA release was also observed in Percoll-purified synaptosomes. Enhanced [$^3$H]DA release is abolished in the presence of NMDAR antagonist D-AP5, but is unaffected by the AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). A modest reduction in PS stimulated [$^3$H]DA release was observed in the presence of an antagonist mixture containing blockers of AMPA/kainate CNQX, 5-$HT_3$ [3-tropanylindole-3-carboxylate hydrochloride (ICS 205,930)], $GABA_A$ (bicuculline), nicotinic acetylcholine (mecamylamine), and DA $D_1$ (SCH23390) and $D_2$-like receptors (sulpiride) (Fig. 4b). Although this effect did not attain statistical significance ($p$ = 0.15 vs. PS alone), it could reflect a small contribution of receptor types other than NMDA receptors to PS-induced [$^3$H]DA release. Taken together, however, these results argue that the effect of PS is primarily mediated via NMDAR activity.

The overflow of [$^3$H]DA is a result of a balance between the rate of efflux of [$^3$H]DA from SSNs and the rate of influx of [$^3$H]DA via re-uptake. The observed PS-induced increase in [$^3$H]DA overflow from striatal SSNs could be the result of stimulation of [$^3$H]DA release and/or inhibition of [$^3$H]DA uptake. To determine whether PS inhibits [$^3$H]DA uptake, we examined the effect of 25 nM PS on [$^3$H]DA uptake kinetics in striatal SSNs. We found no effect of 25 nM PS on initial rate of [$^3$H]DA uptake (Fig. 5), providing evidence that PS does not act via inhibition of re-uptake of DA, but rather acts directly on DA release mechanisms.

The thiol-containing glutathione precursor NAC was included in the superfusion buffer in some experiments to reduce the level of spontaneous [$^3$H]DA release. The presence of this compound had no effect on PS-induced [$^3$H]DA release. Net PS-induced [$^3$H]DA release is 32 ± 4% ($n$ = 4) in the presence of NAC, while the effect size is 36 ± 5% ($n$ = 10) in the absence of NAC.

Pregnenolone sulfate evokes [$^3$H]DA release in a dose-dependent manner with significant evoked release at PS concentrations as low as 25 pM (Fig. 6). PS application does not enhance the release of [$^3$H]GABA or [$^{14}$C]glutamate in double label experiments (Fig. 7), indicating that the effect of PS is specific to dopaminergic terminals.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

## Discussion

Neuroactive steroids exhibit the capacity to modulate ionotropic glutamate and $GABA_A$ receptors in various preparations including neuronal cell culture, expressed receptors in *Xenopus* oocytes, and slices of hippocampal formation (Gibbs *et al.* 2006) but the question of whether these are pharmacological or physiological effects remains to be resolved. PS fulfills many of the criteria for neurotransmitter/neuromodulator function, but a physiological role has not been identified.

Isolated presynaptic terminals represent a model system for investigating the molecular mechanisms underlying presynaptic phenomena (Dunkley *et al.* 1988). Specifically, this preparation is comprised of detached synapses that possess (i) a sealed plasma membrane, (ii) one or more mitochondria, (iii) synaptic vesicles, and (iv) a postsynaptic adhesion (Fig. 1). The striatum is the major target of innervation by DA neurons located in the substantia nigra and DA release is critical for the regulation of voluntary movement. Parkinson's disease results from the specific, progressive degeneration of striatal DA neurons. The striatum is also involved in procedural learning, and changes in striatal activity are implicated in neuropsychiatric disorders including depression and drug addiction (Graybiel 2000). Elucidation of the mechanisms of action of endogenous neurosteroids in this nucleus could lead to approaches for therapeutic intervention in pathologies related to both mood and movement.

Here, we demonstrate that PS at ≥ 25 pM evokes significant release of newly accumulated [$^3$H]DA from a mixed preparation of synaptosomes and synaptoneurosomes. 25 fM PS is virtually without effect on [$^3$H]DA release indicating that the $EC_{50}$ for PS is in the high fM to low pM range. The present results are consistent with those of Sadri-Vakili *et al.* (2008), who found that 10 nM PS, infused into the striatum by *in vivo* reverse microdialysis, enhanced DA overflow, and that this effect of PS was sensitive to D-AP5.

Inhibition of PS-induced [$^3$H]DA release by D-AP5 suggests that PS may enhance DA release by potentiating glutamate activation of presynaptic NMDA receptors located on nigro-striatal dopaminergic terminals. The question of whether release of DA from nigro-striatal dopaminergic terminals is regulated by presynaptic NMDA receptors is controversial. NMDA induces a specific, $Mg^{2+}$ sensitive increase in the release of [$^3$H]DA (Snell and Johnson 1986) or endogenous dopamine (Clow and Jhamandas 1989) from superfused rat striatal slices with an $EC_{50}$ of approximately 60 μM. Multiple investigators have reported specific NMDA-induced release of DA from striatal synaptosomes (Johnson and Jeng 1991; Krebs *et al.* 1991; Wang 1991; Pittaluga *et al.* 2001), which we also observed in the present study. Unilateral 6-hydroxydopamine (6-OHDA) lesions of nigrostriatal dopaminergic neurons in the rat resulted in a small but significant reduction in radioligand binding to NMDA, AMPA, and kainate receptors in the striatum relative to unlesioned contralateral tissue (Tarazi *et al.* 1998). However, immunolabeling studies of sections of rat striatum have failed to detect NMDARs associated with the presynaptic membrane, arguing that presynaptic NMDARs, if present, must be at a lower density than on the postsynaptic membrane (Bernard and Bolam 1998; Gracy *et al.* 1999).

Another possibility is that PS could act indirectly to trigger the release of some other factor that acts upon dopaminergic SSNs to promote [$^3$H]DA release. For example, PS could stimulate release of glutamate from glutamatergic SSNs, which could act upon non-NMDA glutamate receptors on presynaptic DA terminals to stimulate release. This explanation seems unlikely as we did not observe any effect of PS on [$^{14}$C]glutamate release. Moreover, PS-induced [$^3$H]DA release was not significantly reduced by an antagonist mixture that included inhibitors of $GABA_A$, 5-$HT_3$, nicotinic Ach, AMPA/kainate, and dopamine $D_1$ and $D_2$ receptors (Fig. 4b),

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

indicating that these receptors do not play a major role in PS-induced [$^3$H]DA release. It remains conceivable that PS could act via postsynaptic NMDARs on the subpopulation of synaptoneurosomes to stimulate the release of a factor that acts in a retrograde manner to enhance release of [$^3$H]DA from the presynaptic terminal. We did not, however, observe any effect of PS on the release of either [$^{14}$C]glutamate or [$^3$H]GABA, arguing that a retrograde mechanism of this type would have to be specific for dopaminergic synapses.

Pregnenolone sulfate stimulates striatal [$^3$H]DA release at much lower concentrations than expected from studies of PS modulation of NMDARs expressed in *Xenopus* oocytes. PS enhances the activation of NMDARs containing NR2A or NR2B subunits, while inhibiting NMDARs containing NR2C or NR2D subunits, but the $EC_{50}$ for these modulatory effects is in the micromolar range (Malayev *et al.* 2002). PS modulation of spontaneously occurring EPSCs of hippocampal neurons in culture (Park-Chung *et al.* 1997) also requires micromolar concentrations of PS. However, it is clear that PS modulates NMDAR mediated neurotransmission by multiple mechanisms. PS acts presynaptically to enhance glutamate release from CA1 pyramidal neurons in rat hippocampal slices, as measured by enhancement of NMDAR-dependent paired pulse facilitation, with an $EC_{50}$ between 0.1–1 μM (Partridge and Valenzuela 2001). Superfusion of rat hippocampal slices with 300 nM PS specifically enhances NMDAR dependent long term potentiation in the CA1 region (Sliwinski *et al.* 2004). PS, at concentrations between 0.1 and 10 nM, enhances the binding of [$^3$H]ifenprodil to NMDARs containing the NR2B subunit (Johansson *et al.* 2008). Furthermore, intracerebroventricular administration of subnanomolar concentrations of PS increases extracellular DA levels in the nucleus accumbens (Barrot *et al.* 1999) and reduces learning impairment induced by antagonists of NMDARs and muscarinic receptors (Mathis *et al.* 1996; Meziane *et al.* 1996). At present, it is unclear whether the high potency modulatory effect of PS on [$^3$H]DA release from SSNs is mediated through the same binding site(s) that are responsible for the low affinity modulation of NMDA-induced currents in cultured neurons and expressed receptors or whether an additional site of activity may be involved. The details of the mechanism whereby high affinity interactions of PS with NMDA receptors couple to enhancement DA release remain to be elucidated.

The present results indicate that PS is able to modulate DA release in the striatum at concentrations in the low pM range, a concentration range that is similar to measured tissue levels of PS in rat brain (Liere *et al.* 2004). This evidence is consistent with a potential physiological role for PS in modulation of DA release from striatal synaptic terminals. As suggested by Schumacher *et al.* (2008), high local PS concentrations may exist in the brain, representing a possible physiological role for low affinity effects of PS on neurotransmission.

The present study provides evidence that PS is capable of inducing [$^3$H]DA release at concentrations comparable to those at which other steroids can interact with classical intracellular steroid receptors. For example, estradiol induces estrogen response element mediated luciferase activity in MCF-7 (human breast cancer) cells with an $EC_{50}$ of 30 pM (Chang *et al.* 2007). The tissue content of estradiol in the rodent brain corresponds to average concentrations of 0.6–1.2 nM (Morissette *et al.* 1992; Hojo *et al.* 2004) while the levels in human brain are 0.84– 4.0 nM (Lanthier and Patwardhan 1986). Like PS, estradiol exhibits potent neuromodulatory effects. Estradiol rapidly (60 min) augments KCl-stimulated (Becker 1990) and amphetamine-stimulated (Xiao *et al.* 2003) release of DA from striatal tissue of ovariectomized rats at a concentration of 370 pM. Furthermore, estradiol has been shown to influence synaptic plasticity at concentrations of 0.1–10 nM (Foy *et al.* 1999; Ito *et al.* 1999; Kow *et al.* 2005; Mukai *et al.* 2007).

It is unlikely that residual endogenous extracellular PS would be able to influence transmitter release from SSNs under the experimental conditions used in this study. Extracellular striatal

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

PS would be expected to be eliminated during the homogenization, centrifugation, and resuspension steps required for SSN preparation. Additionally, the small amount of SSNs are layered onto microporous glass fiber filters and continuously perfused with aCSF throughout experimentation. While the possibility exists that PS may be synthesized and released locally at synaptic terminals, our observation of significant enhancement of [$^3$H]DA release upon application of pM PS argues against the release and accumulation of significant levels of endogenous PS in perfused striatal SSNs.

The results described in this report demonstrate that subnanomolar concentrations of PS enhance DA release from striatal SSNs via an NMDAR-dependent mechanism, supporting the hypothesis that PS may function as an endogenous modulator of dopaminergic neurotransmission. Changes in DA levels because of modulation of excitatory transmission by PS may alter motor activity, mood, motivation, and reward pathways. Increased DA transmission in the striatal complex is linked to the etiology of schizophrenia and Tourette's syndrome, among other disorders (Konradi and Heckers 2003; Saka and Graybiel 2003) raising the possibility that defects in PS modulation of DA release could contribute to the development of neurological and psychiatric disorders. Conversely, developing compounds that influence PS modulation may provide a novel strategy for the development of therapeutics for diseases involving defects in dopaminergic neurotransmission.

## Acknowledgments

This work was supported by NIMH R01 MH-049469. The authors would like to thank Dr. Alan Peters, Dr. Haiyan Gong, and Rozanne Richman for their generous assistance with the EM studies.

## References

Barrot M, Vallee M, Gingras MA, Le Moal M, Mayo W, Piazza PV. The neurosteroid pregnenolone sulphate increases dopamine release and the dopaminergic response to morphine in the rat nucleus accumbens. Eur J Neurosci 1999;11:3757–3760. [PubMed: 10564382]

Becker JB. Direct effect of 17 beta-estradiol on striatum: sex differences in dopamine release. Synapse 1990;5:157–164. [PubMed: 2309159]

Bernard V, Bolam JP. Subcellular and subsynaptic distribution of the NR1 subunit of the NMDA receptor in the neostriatum and globus pallidus of the rat: co-localization at synapses with the GluR2/3 subunit of the AMPA receptor. Eur J Neurosci 1998;10:3721–3736. [PubMed: 9875351]

Bukusoglu C, Sarlak F. Pregnenolone sulfate increases intracellular Ca2+ in a pituitary cell line. Eur J Pharmacol 1996;298:79–85. [PubMed: 8867923]

Chang M, Peng KW, Kastrati I, Overk CR, Qin ZH, Yao P, Bolton JL, Thatcher GR. Activation of estrogen receptor-mediated gene transcription by the equine estrogen metabolite, 4-methoxyequilenin, in human breast cancer cells. Endocrinology 2007;148:4793–4802. [PubMed: 17584965]

Clow DW, Jhamandas K. Characterization of L-glutamate action on the release of endogenous dopamine from the rat caudate-putamen. J Pharmacol Exp Ther 1989;248:722–728. [PubMed: 2563769]

Compagnone NA, Salido E, Shapiro LJ, Mellon SH. Expression of steroid sulfatase during embryogenesis. Endocrinology 1997;138:4768–4773. [PubMed: 9348204]

Dong Y, Fu YM, Sun JL, Zhu YH, Sun FY, Zheng P. Neurosteroid enhances glutamate release in rat prelimbic cortex via activation of alpha1-adrenergic and sigma1 receptors. Cell Mol Life Sci 2005;62:1003–1014. [PubMed: 15798892]

Dunkley PR, Heath JW, Harrison SM, Jarvie PE, Glenfield PJ, Rostas JA. A rapid Percoll gradient procedure for isolation of synaptosomes directly from an S1 fraction: homogeneity and morphology of subcellular fractions. Brain Res 1988;441:59–71. [PubMed: 2834006]

Ebner MJ, Corol DI, Havlìková H, Honour JW, Fry JP. Identification of neuroactive steroids and their precursors and metabolites in adult male rat brain. Endocrinology 2006;147:179–190. [PubMed: 16223859]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Farb, DH.; Gibbs, TT. Steroids as modulators of amino acid receptor function. In: Stone, TW., editor. CNS Transmitters and Neuromodulators: Neuroactive Steroids. CRC Press; New York: 1996. p. 23-36.

Foy MR, Xu J, Xie X, Brinton RD, Thompson RF, Berger TW. 17beta-estradiol enhances NMDA receptor-mediated EPSPs and long-term potentiation. J Neurophysiol 1999;81:925–929. [PubMed: 10036289]

Gibbs TT, Farb DH. Dueling enigmas: neurosteroids and sigma receptors in the limelight. Sci STKE 2000;2000:PE1. [PubMed: 11752623]

Gibbs TT, Russek SJ, Farb DH. Sulfated steroids as endogenous neuromodulators. Pharmacol Biochem Behav 2006;84:555–567. [PubMed: 17023038]

Gracy KN, Clarke CL, Meyers MB, Pickel VM. N-methyl-D-aspartate receptor 1 in the caudate-putamen nucleus: ultrastructural localization and co-expression with sorcin, a 22,000 mol. wt calcium binding protein. Neuroscience 1999;90:107–117. [PubMed: 10188938]

Graybiel AM. The basal ganglia. Curr Biol 2000;10:R509–R511. [PubMed: 10899013]

Hayashi T, Maurice T, Su TP. $Ca^2\zeta$ signaling via σ-receptors: novel regulatory mechanism affecting intracellular $Ca^2\zeta$ concentration. J Pharmacol Exp Ther 2000;293:788–798. [PubMed: 10869377]

Hige T, Fujiyoshi Y, Takahashi T. Neurosteroid pregnenolone sulfate enhances glutamatergic synaptic transmission by facilitating presynaptic calcium currents at the calyx of Held of immature rats. Eur J Neurosci 2006;24:1955–1966. [PubMed: 17040476]

Hojo Y, Hattori TA, Enami T, et al. Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017alpha and P450 aromatase localized in neurons. Proc Natl Acad Sci USA 2004;101:865–870. [PubMed: 14694190]

Ito K, Skinkle KL, Hicks TP. Age-dependent, steroid-specific effects of oestrogen on long-term potentiation in rat hippocampal slices. J Physiol 1999;515:209–220. [PubMed: 9925890]

Johansson T, Frändberg PA, Nyberg F, Le Grevès P. Molecular mechanisms for nanomolar concentrations of neurosteroids at NR1/NR2B receptors. J Pharmacol Exp Ther 2008;324:759–768. [PubMed: 18006693]

Johnson KM, Jeng YJ. Pharmacological evidence for N-methyl-D-aspartate receptors on nigrostriatal dopaminergic nerve terminals. Can J Physiol Pharmacol 1991;69:1416–1421. [PubMed: 1685693]

Kohjitani A, Fuda H, Hanyu O, Strott CA. Cloning, characterization and tissue expression of rat SULT2B1a and SULT2B1b steroid/sterol sulfotransferase isoforms: divergence of the rat SULT2B1 gene structure from orthologous human and mouse genes. Gene 2006;367:66–73. [PubMed: 16368200]

Kohjitani A, Fuda H, Hanyu O, Strott CA. Regulation of SULT2B1a (pregnenolone sulfotransferase) expression in rat C6 glioma cells: relevance of AMPA receptor-mediated NO signaling. Neurosci Lett 2008;430:75–80. [PubMed: 18054434]

Konradi C, Heckers S. Molecular aspects of glutamate dysregulation: implications for schizophrenia and its treatment. Pharmacol Ther 2003;97:153–179. [PubMed: 12559388]

Kow LM, Easton A, Pfaff DW. Acute estrogen potentiates excitatory responses of neurons in rat hypothalamic ventromedial nucleus. Brain Res 2005;1043:124–131. [PubMed: 15862525]

Krebs MO, Desce JM, Kemel ML, Gauchy C, Godeheu G, Cheramy A, Glowinski J. Glutamatergic control of dopamine release in the rat striatum: evidence for presynaptic N-methyl-D-aspartate receptors on dopaminergic nerve terminals. J Neurochem 1991;56:81–85. [PubMed: 1824785]

Lanthier A, Patwardhan VV. Sex steroids and 5-en-3 beta-hydroxysteroids in specific regions of the human brain and cranial nerves. J Steroid Biochem 1986;25:445–449. [PubMed: 2945971]

Liere P, Pianos A, Eychenne B, Cambourg A, Liu S, Griffiths W, Schumacher M, Sjövall J, Baulieu EE. Novel lipoidal derivatives of pregnenolone and dehydroepiandrosterone and absence of their sulfated counterparts in rodent brain. J Lipid Res 2004;45:2287–2302. [PubMed: 15342680]

Malayev AA, Gibbs TT, Farb DH. Inhibition of the NMDA Response by pregnenolone sulfate reveals subtype selective modulation of NMDA receptors by sulfated steroids. Br J Pharmacol 2002;135:901–909. [PubMed: 11861317]

Mameli M, Carta M, Partridge LD, Valenzuela CF. Neurosteroid-induced plasticity of immature synapses via retrograde modulation of presynaptic NMDA receptors. J Neurosci 2005;25:2285–2294. [PubMed: 15745954]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Mathis C, Vogel E, Cagniard B, Criscuolo F, Ungerer A. The neurosteroid pregnenolone sulfate blocks deficits induced by a competitive NMDA antagonist in active avoidance and lever-press learning tasks in mice. Neuropharmacology 1996;35:1057–1064. [PubMed: 9121608]

Mellon SH, Griffin LD, Compagnone NA. Biosynthesis and action of neurosteroids. Brain Res Brain Res Rev 2001;37:3–12. [PubMed: 11744070]

Meyer DA, Carta M, Partridge LD, Covey DF, Valenzuela CF. Neurosteroids enhance spontaneous glutamate release in hippocampal neurons. Possible role of metabotropic r-like receptors. J Biol Chem 2002;277:28725–28732. [PubMed: 12042305]

Meziane H, Mathis C, Paul SM, Ungerer A. The neurosteroid pregnenolone sulfate reduces learning deficits induced by scopolamine and has promnestic effects in mice performing an appetitive learning task. Psychopharmacology (Berl) 1996;126:323–330. [PubMed: 8878348]

Monnet FP, Mahe V, Robel P, Baulieu EE. Neurosteroids, via σ receptors, modulate the [$^{3}$H] norepinephrine release evoked by N-methyl-D-aspartate in the rat hippocampus. Proc Natl Acad Sci USA 1995;92:3774–3778. [PubMed: 7731982]

Morissette M, Garcia-Segura LM, Belanger A, DiPaolo T. Changes of rat striatal neuronal membrane morphology and steroid content during the estrous cycle. Neuroscience 1992;49:893–902. [PubMed: 1436487]

Mukai H, Tsurugizawa T, Murakami G, et al. Rapid modulation of long-term depression and spinogenesis via synaptic estrogen receptors in hippocampal principal neurons. J Neurochem 2007;100:950–967. [PubMed: 17266735]

Park-Chung M, Wu F, Purdy RH, Malayev AA, Gibbs TT, Farb DH. Distinct sites for inverse modulation of NMDA receptors by sulfated steroids. Mol Pharmacol 1997;52:1113–1123. [PubMed: 9396781]

Partridge LD, Valenzuela CF. Neurosteroid-induced enhancement of glutamate transmission in rat hippocampal slices. Neurosci Lett 2001;301:103–106. [PubMed: 11248433]

Pittaluga A, Pattarini R, Feligioni M, Raiteri M. N-methyl-D-aspartate receptors mediating hippocampal noradrenaline and striatal dopamine release display differential sensitivity to quinolinic acid, the HIV-1 envelope protein gp120, external pH and protein kinase C inhibition. J Neurochem 2001;76:139–148. [PubMed: 11145986]

Plassart-Schiess E, Baulieu EE. Neurosteroids: recent findings. Brain Res Brain Res Rev 2001;37:133–140. [PubMed: 11744081]

Sabeti J, Nelson TE, Purdy RH, Gruol DL. Steroid pregnenolone sulfate enhances NMDA-receptor-independent long-term potentiation at hippocampal CA1 synapses: role for L-type calcium channels and sigma-receptors. Hippocampus 2007;17:349–369. [PubMed: 17330865]

Sadri-Vakili G, Janis G, Pierce R, Gibbs TT, Farb DH. Nanomolar concentrations of pregnenolone sulfate enhance striatal dopamine overflow *in vivo*. J Pharmacol Exp Ther. 2008in press

Saka E, Graybiel AM. Pathophysiology of Tourette's syndrome: striatal pathways revisited. Brain Dev 2003;25(Suppl 1):S15–S19. [PubMed: 14980366]

Sanz AG, Hospital S, Badia A, Clos MV. Presynaptic effect of 7-OH-DPAT on evoked [3H]acetylcholine release in rat striatal synaptosomes. Brain Res 2000;874:116–122. [PubMed: 10960595]

Schiess AR, Scullin CS, Partridge LD. Neurosteroid-induced enhancement of short-term facilitation involves a component downstream from presynaptic calcium in hippocampal slices. J Physiol 2006;576:833–847. [PubMed: 16931546]

Schumacher M, Liere P, Akwa Y, Rajkowski K, Griffiths W, Bodin K, Sjövall J, Baulieu EE. Pregnenolone sulfate in the brain: a controversial neurosteroid. Neurochem Int 2008;52:522–540. [PubMed: 18068870]

Sliwinski A, Monnet FP, Schumacher M, Morin-Surun MP. Pregnenolone sulfate enhances long-term potentiation in CA1 in rat hippocampus slices through the modulation of N-methyl-D-aspartate receptors. J Neurosci Res 2004;78:691–701. [PubMed: 15505794]

Snell LD, Johnson KM. Characterization of the inhibition of excitatory amino acid-induced neurotransmitter release in the rat striatum by phencyclidine-like drugs. J Pharmacol Exp Ther 1986;238:938–946. [PubMed: 2875174]

Tarazi FI, Campbell A, Yeghiayan SK, Baldessarini RJ. Localization of ionotropic glutamate receptors in caudate-putamen and nucleus accumbens septi of rat brain: comparison of NMDA, AMPA, and kainate receptors. Synapse 1998;30:227–235. [PubMed: 9723793]

Wang JK. Presynaptic glutamate receptors modulate dopamine release from striatal synaptosomes. J Neurochem 1991;57:819–822. [PubMed: 1650394]

Wu FS, Gibbs TT, Farb DH. Pregnenolone sulfate: a positive allosteric modulator at the N-methyl-D-aspartate receptor. Mol Pharmacol 1991;40:333–336. [PubMed: 1654510]

Xiao L, Jackson LR, Becker JB. The effect of estradiol in the striatum is blocked by ICI 182,780 but not tamoxifen: pharmacological and behavioral evidence. Neuroendocrinology 2003;77:239–245. [PubMed: 12766324]

## Abbreviations

**aCSF**
: artificial cerebrospinal fluid

**AMPA**
: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

**DA**
: dopamine

**D-AP5**
: DL-2-amino-5-phosphonovaleric acid

**DMSO**
: dimethylsulfoxide

**EPSC**
: excitatory postsynaptic current

**NAC**
: *n*-acetylcysteine

**NMDAR**
: NMDA receptor

**PS**
: pregnenolone sulfate

**SSN**
: synaptosome/synaptoneurosome



NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

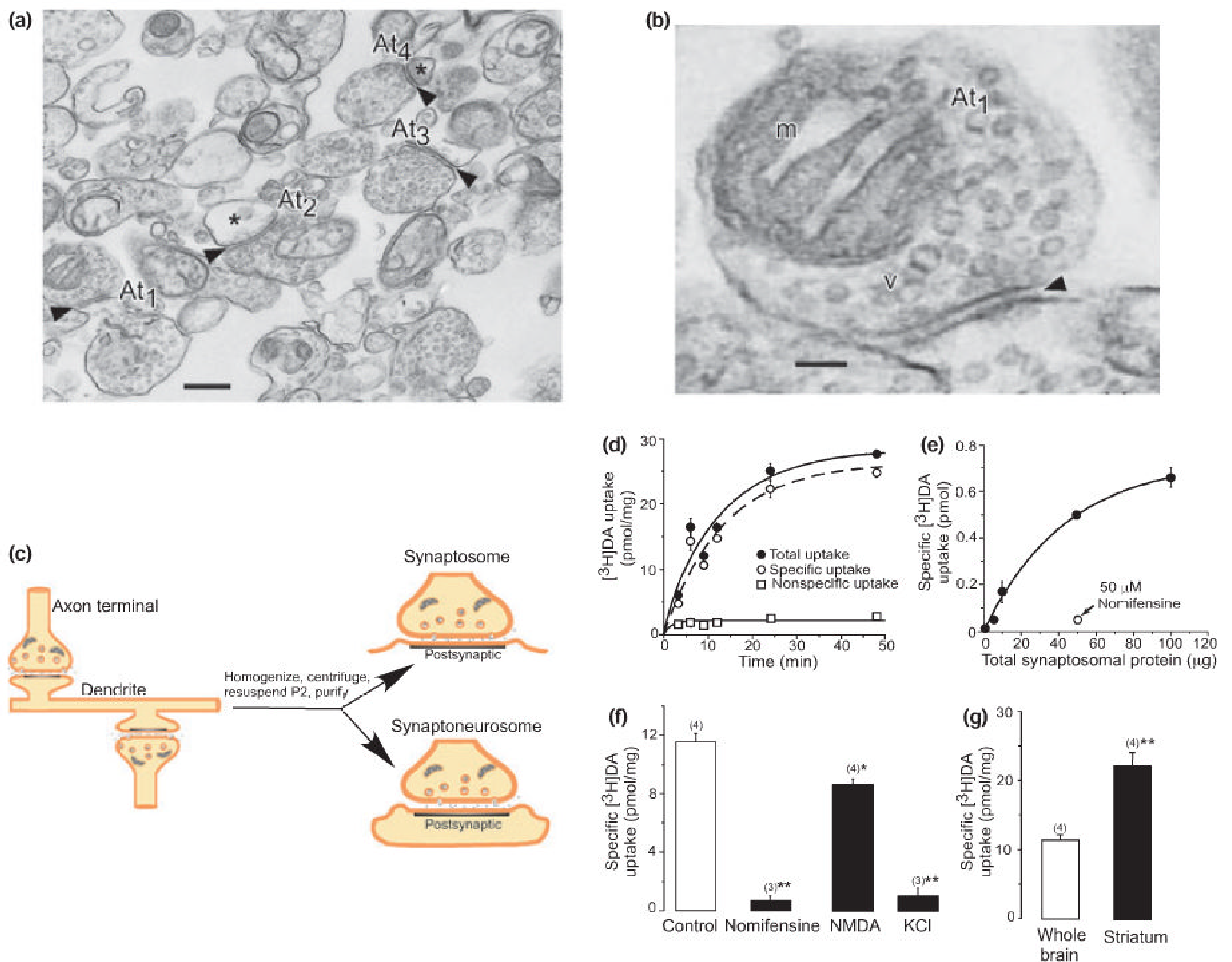


**Fig. 1.**

Purified striatal axon terminals contain a mixture of synaptosomes and synaptoneurosomes (SSNs) capable of specific neurotransmitter uptake. (a) Electron micrograph of a representative section through the SSN pellet demonstrates intact axon terminals ($At_1$–$At_4$). Intact symmetric synaptic junctions with attached postsynaptic membranes are indicated by arrowheads. The preparation contains a mixture of synaptosomes ($At_1$ & $At_3$) and synaptoneurosomes ($At_2$ & $At_4$). Asterisks indicate the intact postsynaptic element that is present in synaptoneurosomes. The electron micrograph shows a representative section through the SSN pellet and does not represent the SSN preparation as it would appear on a superfusion filter during experimentation. Scale bar = 500 nm. (b) Higher magnification shows an individual synaptosome ($At_1$) containing a mitochondrion (m), numerous synaptic vesicles (v), and a synaptic junction (tip of the arrowhead). Scale bar = 100 nm. SSNs were prepared for electron microscopy using $Mg^{2+}$-free aCSF. This buffer was used in all sub-sequent neurotransmitter uptake and superfusion (see Materials and Methods) experiments. (c) Schematic illustration of the preparation of fractionated axon terminals and their postsynaptic elements. The final suspension contains a mixture of both synaptosomes and synaptoneurosomes. (d–g) Newly accumulated [$^3$H]DA is transported and packaged into a releasable form by striatal SSNs. (d) The kinetics of specific [$^3$H]DA uptake (dotted line) are fit by a single exponential, indicative

of a single component transport system. (e) Specific uptake of [$^3$H]DA increases with protein concentration and is blocked by the specific DA transport inhibitor nomifensine. (f) Specific [$^3$H]DA accumulation is reduced when uptake is carried out in the presence of nomifensine (50 μM), NMDA (100 μM), or KCl (100 mM). (g) Specific [$^3$H]DA uptake is two-fold greater in striatal SSNs as compared with whole brain SSNs. *$p < 0.05$; **$p < 0.01$ (unpaired *t*-test). Values above error bars indicate the number of replicate experiments carried out for each condition.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

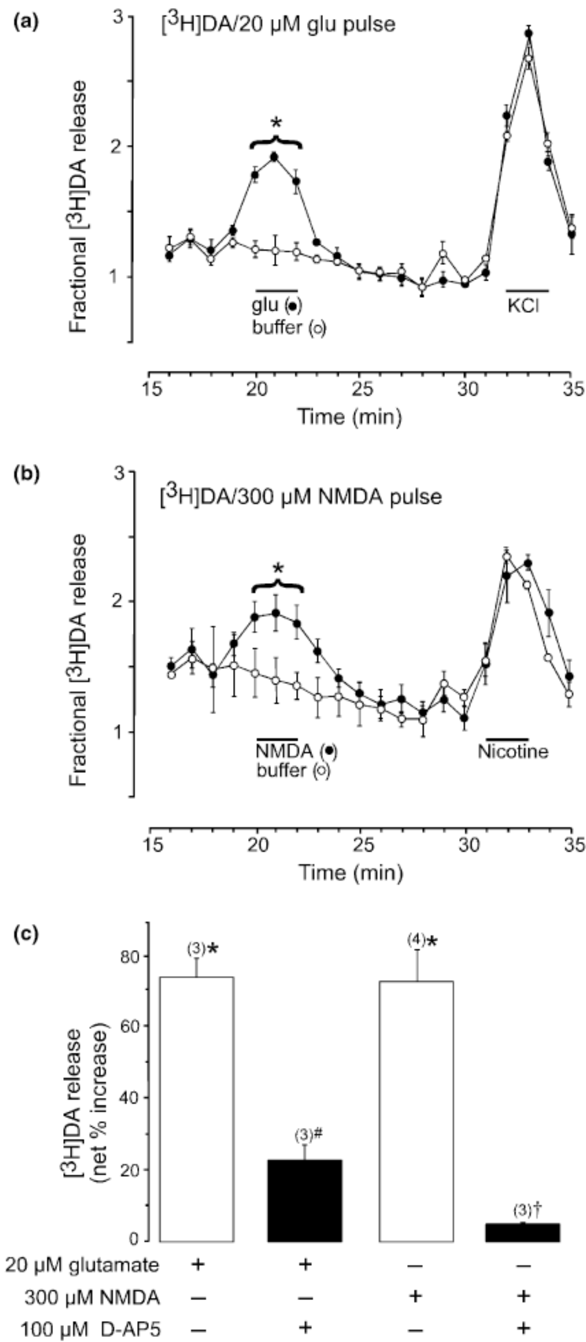


**Fig. 2.**

Glutamate, NMDA, KCl, and nicotine induce the release of newly accumulated [$^3$H]DA from striatal SSNs. Each experiment was comprised of six superfusion trials carried out in parallel. At t = 20 min three samples received a 2 min test pulse of agonist + 1 μM glycine (filled circles); while three samples received a pulse of buffer alone (open circles). All six samples received a second 2 min pulse of either 10 mM KCl or 500 μM nicotine. (a) 20 μM glutamate + 1 μM glycine (glu) application induces [$^3$H]DA release from striatal SSNs. A second pulse of 10 mM KCl increases [$^3$H]DA release from both agonisttreated and buffer-treated SSNs by a similar amount. (b) 300 μM NMDA + 1 μM glycine (NMDA) application induces [$^3$H]DA release from striatal SSNs. A second pulse of 500 μM nicotine induces [$^3$H]DA release from both

agonist treated and buffer treated SSNs to a similar degree. Each data point represents the mean ± SEM values of three (a) or four (b) independent experiments. (c) Stimulation of [$^3$H]DA release by glutamate + glycine or NMDA + glycine is inhibited by the NMDA receptor antagonist D-AP5. Net release values were calculated as described in Materials and Methods. *$p < 0.02$ compared to buffer control (paired *t*-test); #$p = 0.005$ compared to glutamate + glycine (unpaired *t*-test); †$p = 0.005$ compared to NMDA + glycine (unpaired *t*-test). Values above error bars indicate the number of replicate experiments carried out for each condition.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

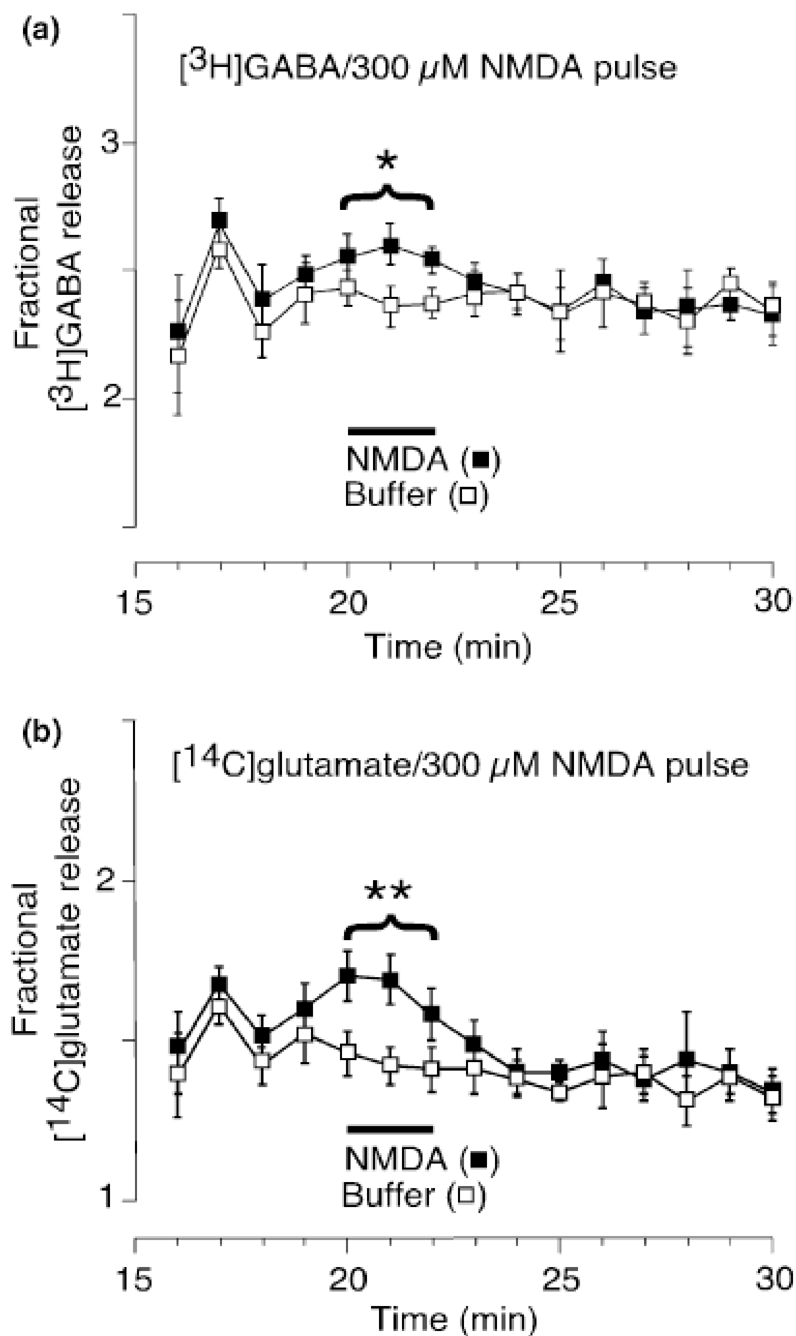


**Fig. 3.**

300 μM NMDA + 1 μM glycine (NMDA) application induces the release of newly accumulated [$^3$H]GABA and [$^{14}$C]glutamate from striatal SSNs. Each experiment was comprised of six superfusion trials carried out in parallel. At t = 20 min three samples received a 2 min test pulse of 300 μM NMDA + 1 μM glycine (filled squares); while three samples received a pulse of buffer alone (open squares). (a) 2 min NMDA + glycine application induces statistically significant release of [$^3$H]GABA from striatal SSNs. (b) 2 min NMDA + glycine application induces statistically significant release of [$^{14}$C]glutamate from striatal SSNs. *$p < 0.05$ (paired *t*-test), $n = 4$; **$p = 0.01$ (paired *t*-test), $n = 4$.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

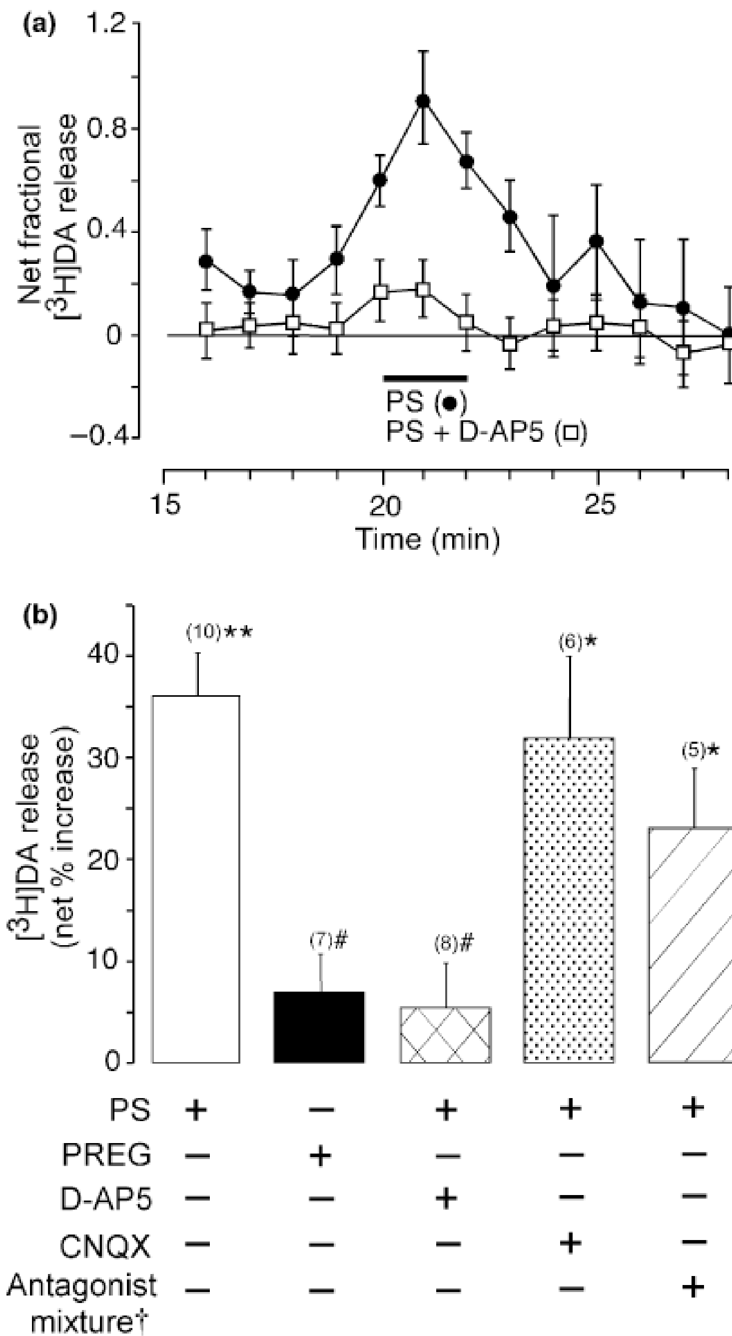


**Fig. 4.**

PS-induced [$^3$H]DA release from striatal SSNs is NMDAR dependent. (a) Net [$^3$H]DA release from striatal SSNs elicited by a 2-min pulse of either 25 nM PS or 25 nM PS + 100 μM D-AP5. Each experiment was comprised of six superfusion trials carried out in parallel. *Closed circles*: at t = 20 min three samples received a 2-min test pulse of 25 nM PS while three samples received a pulse of buffer alone. Net fractional release values for each time point were obtained by subtracting the release values for buffer alone treated SSNs from the release values for PS treated SSNs ($n$ = 10). Open squares: at t = 20 min three samples received a 2-min test pulse of 25 nM PS + 100 μM D-AP5 while three samples received a pulse of 100 μM D-AP5 alone. Net fractional release values for each time point were obtained by subtracting the release values

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

for D-AP5 alone treated SSNs from the release values for PS + D-AP5 treated SSNs ($n = 8$). (b) Specific release of [$^3$H]DA in response to a pulse of 25 nM PS is not significantly inhibited by blocking AMPA receptors with 10 μM CNQX ($p = 0.47$ vs. PS alone, unpaired *t*-test) or by a mixture of inhibitors for AMPARs, 5-hydroxytryptamine$_3$ receptors (5-HT$_3$Rs), nicotinic acetylcholine receptors (nAchRs), GABA$_A$Rs, D$_1$Rs, and D$_2$Rs (CNQX, 10 μM; ICS 205, 930, 5 nM; mecamylamine, 10 μM; bicuculline 5 μM; SCH23390, 1 μM and sulpiride, 1 μM; $p = 0.15$ vs. PS alone, unpaired *t*-test). In contrast, the NMDAR antagonist D-AP5 (100 μM) significantly inhibited 25 nM PS-induced [$^3$H]DA release. 25 nM pregnenolone (PREG) did not induce significant [$^3$H]DA release ($p = 0.16$ vs. control treated SSNs, paired *t*-test). Net release values were calculated as described in Materials and Methods. *$p < 0.05$; **$p = 0.001$ vs. control treated SSNs (paired *t*-test); #$p < 0.001$ vs. PS alone (unpaired *t*-test). Values above error bars indicate thenumber of replicate experiments carried out for each condition.
†Receptors blocked: GABA$_A$R, 5-HT$_3$R, nAchR, D$_1$R, D$_2$R, AMPA/KaR.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

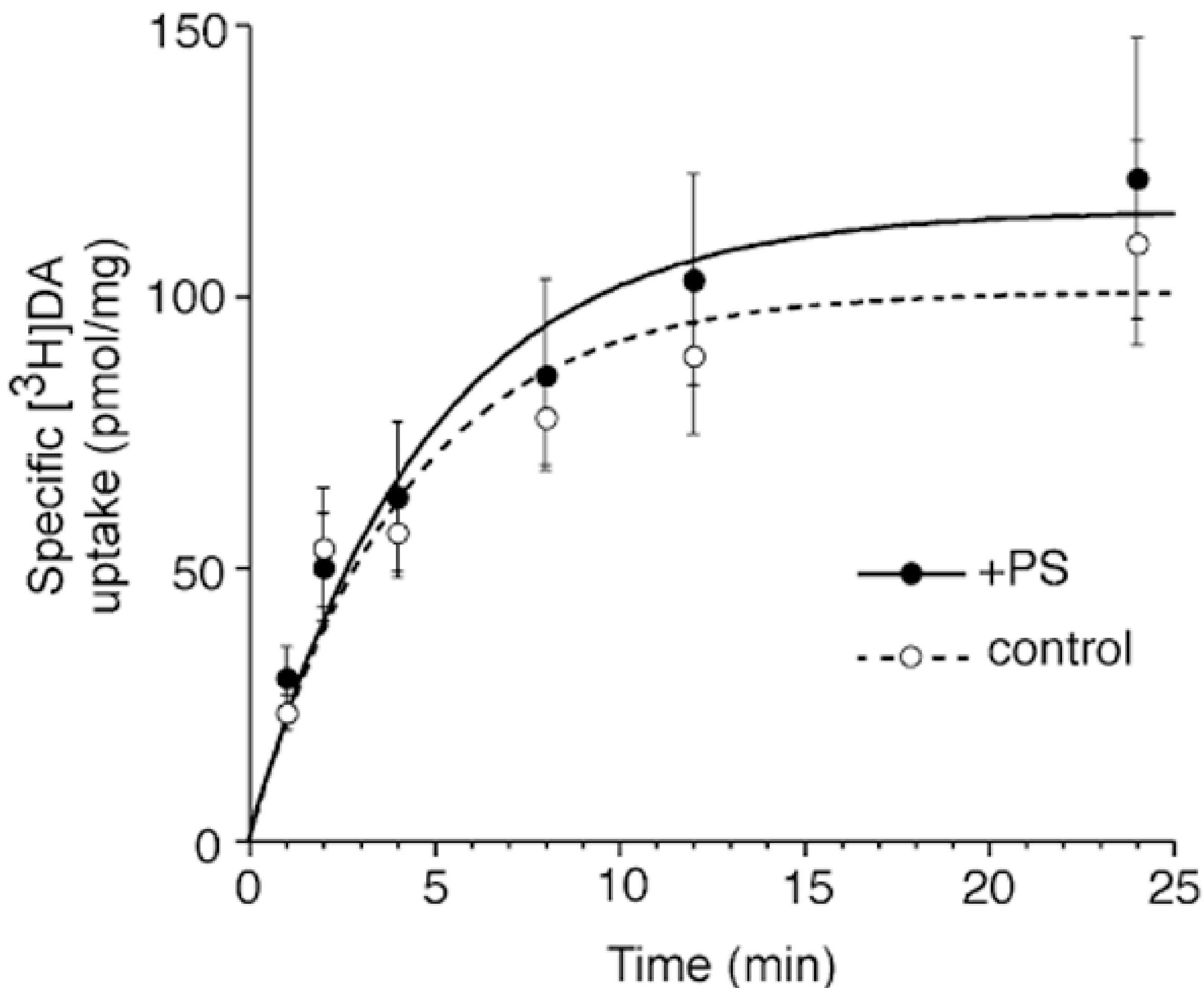


**Fig. 5.**

PS does not influence the initial rate of uptake of [$^3$H]DA into striatal SSNs. Specific uptake of [$^3$H]DA into striatal SSNs was measured in the presence or absence of PS (25 nM). Each data point represents mean ± SEM values from three experiments performed in triplicate. Uptake data was fit to the equation $y = y_{max} \times (1 - e^{-t/\tau})$. Initial slope was calculated for each trial using the equation $slope = y_{max}/\tau$. The initial rate of [$^3$H]DA uptake in the presence of PS was 99.8 ± 12.8% of control ($n = 3$, $p = 0.97$, paired $t$-test) indicating that PS does not inhibit DAT function.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

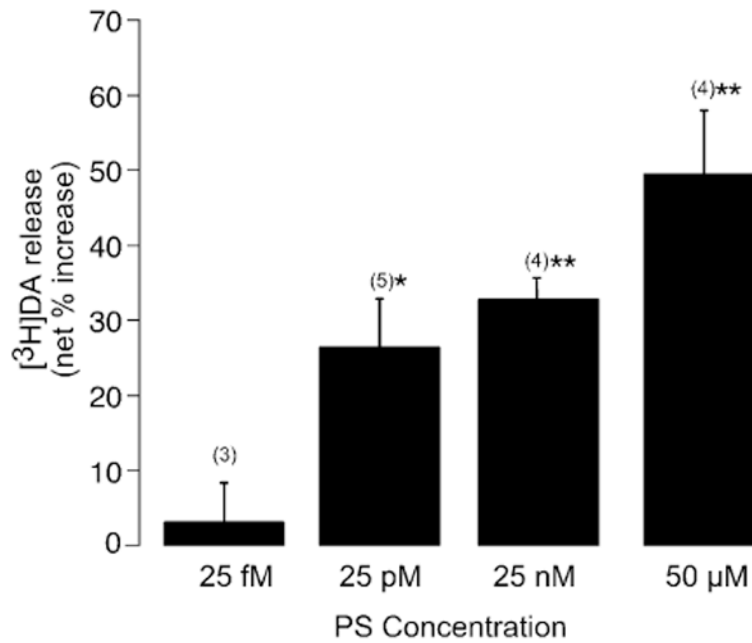


**Fig. 6.**

Dose response relationship for PS-induced increase in [$^3$H]DA release reveals a potency in the picomolar range. 25 fM PS did not increase [$^3$H]DA release relative to control. Evoked [$^3$H]DA release effect was quantified as described in Materials and Methods. These experiments were carried out using superfusion buffer that contained the thiol-containing antioxidant compound *n*-acetylcysteine (NAC, 500 μM). The presence of NAC reduced background [$^3$H]DA release and did not significantly influence the 25 nM PS effect on [$^3$H]DA release (+NAC: 31.6 ± 4.5%, $n = 4$; −NAC: 35.9 ± 5.4%, $n = 10$). *$p < 0.05$; **$p < 0.01$ compared to buffer treated control (paired *t*-test). Values above error bars indicate the number of replicate experiments carried out for each condition.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript



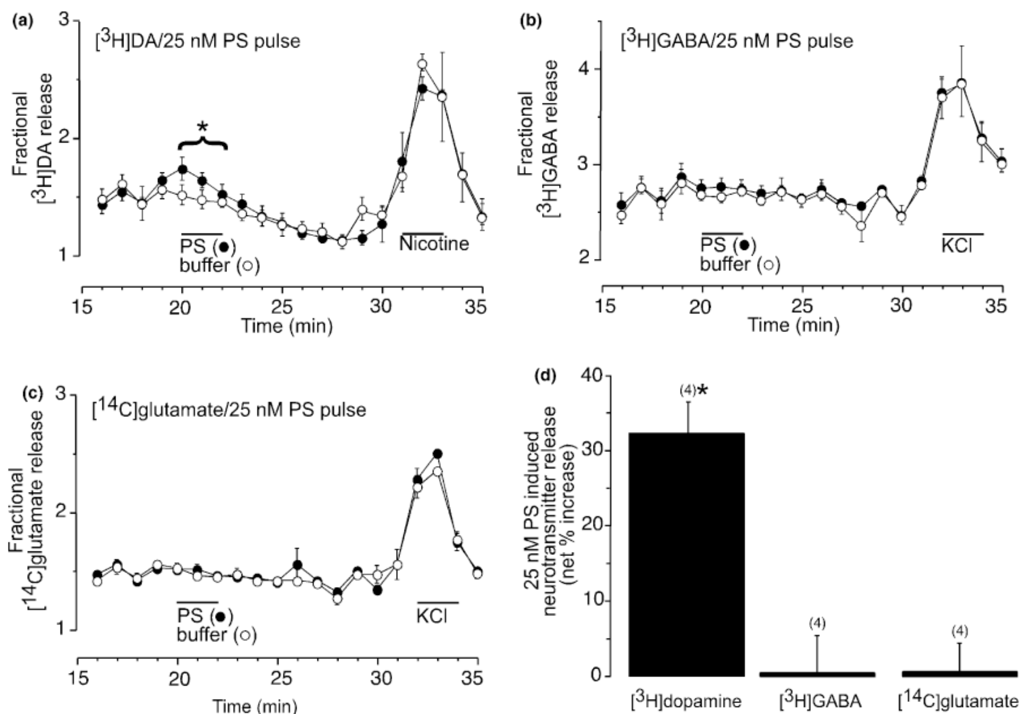


**Fig. 7.**

PS selectively induces the release of newly accumulated [$^3$H]DA from striatal SSNs as compared with [$^3$H]GABA and [$^{14}$C]glutamate. Each experiment was comprised of six superfusion trials carried out in parallel. At t = 20 min three samples received a 2 min test pulse of 25 nM PS (filled circles); while three samples received a pulse of buffer alone (open circles). All six samples were treated with a second 2 min pulse of either 10 mM KCl or 500 μM nicotine. (a) 25 nM PS increases the release of [$^3$H]DA from striatal SSNs. A second pulse of 500 μM nicotine induces [$^3$H]DA release from both PS treated and buffer treated SSNs to a similar degree. In contrast, 25 nM PS had no effect on [$^3$H]GABA (b) or [$^{14}$C]glutamate (c) release. (d) 25 nM PS evokes the release of [$^3$H]DA, but not [$^3$H]GABA or [$^{14}$C]glutamate, from perfused striatal SSNs. Net release values were calculated as described in Materials and Methods. *$p < 0.01$ vs. buffer treated control (paired *t*-test).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

**Table 1**

Specific neurotransmitter uptake in striatal SSNs

| Neurotransmitter | Inhibitor | Uptake inhibition (%) | *n* |
|---|---|---|---|
| [$^{3}$H]dopamine | Nomifensine | 96.1 ± 0.9 | 3 |
| [$^{3}$H]GABA | Nipecotic acid | 96.7 ± 1.5 | 3 |
| [$^{14}$C]glutamate | DL-TBOA | 60.1 ± 5.0 | 4 |

Neurotansmitter uptake into striatal SSNs is inhibited by blockade of specific transport proteins. Striatal SSNs were incubated with radiolabeled neurotransmitter for 15 min at 37°C in the presence or absence of inhibitors of DAT (50 μM nomifensine), GAT1 (100 μM nipecotic acid) or EAAT 1-5 (300 μM DL-TBOA).

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

**Table 2**

Evoked neurotransmitter release (% increase) from striatal SSNs

| | Stimulus | | | |
|---|---|---|---|---|
| **Transmitter released** | **PS (*n*)** | **NMDA (*n*)** | **Nicotine (*n*)** | **KCl (*n*)** |
| [$^3$H]dopamine | 32 ± 4 (4)** | 73 ± 9 (4)** | 226 ± 40 (8)** | 353 ± 20 (4)** |
| [$^{14}$C]glutamate | 1 ± 4 (4) | 30 ± 5 (4)* | 40 ± 8 (4)* | 158 ± 4 (4)** |
| [$^3$H]GABA | 1 ± 5 (4) | 9 ± 2 (4)* | 17 ± 2 (3)* | 137 ± 6 (4)** |

PS = 25 nM; NMDA = 300 μM NMDA + 1 μM glycine; nicotine = 500 μM; KCl = 10 mM. PS and NMDA were applied as S1 pulses. Nicotine and KCl were applied as S2 pulses. Evoked transmitter release was calculated as described in Materials and Methods.

* $p < 0.05$;

** $p < 0.01$.