Brain Research, 186 (1980) 409–419

D-AMPHETAMINE REDUCES STRIATAL SUBSTANCE P CONCENTRATIONS BY PRESYNAPTIC RELEASE OF DOPAMINE

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(Accepted August 9th, 1979)

Key words: substance P — dopamine — striatum — amphetamine — brain peptides

SUMMARY

Single, but not repeated, doses of D-amphetamine sulfate cause dose-dependent reductions in substance P (SP) immunoreactive material within the rat striatum. A 10 mg/kg (i.p.) dose reduces SP levels from 1.37 to 0.97 pmol/10 mg tissue after 2 h. Similar reductions in striatal SP levels are observed after administration of methylphenidate (50 or 100 mg/kg). No changes in SP concentrations occur within the substantia nigra or hypothalamus after D-amphetamine. The amphetamine-induced decline in striatal SP is blocked by pretreating rats with α-methyl tyrosine methyl ester (225 mg/kg i.p.), or haloperidol (1, 3 or 10 mg/kg i.p.), or after the dopaminergic nigrostriatal tract has been lesioned using 6-hydroxydopamine. These data indicate that the mechanism by which D-amphetamine lowers striatal SP involves presynaptic release of dopamine from the terminals of nigrostriatal neurons.

INTRODUCTION

Substance P (SP), a potent neuroactive peptide, is found widely distributed throughout the central and peripheral nervous systems[4,11] and may act as a neurotransmitter (e.g. within primary sensory neurons that convey pain information to the spinal cord[11,30]). There are at least two neural pathways in the brain that contain SP. One, the fasciculus retroflexus, includes axons from SP-containing cell bodies in the medial habenular nucleus en route to the interpeduncular nucleus[12,23]; the other, a striatonigral tract, runs parallel to and in the same neuronal bundle with GABAergic

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neurons[13,16,24]. SP released from the latter neurons may exert an excitatory influence on dopamine-containing neurons in the reciprocal nigrostriatal tract. Thus, microiontophoretic application of SP to perikarya in the substantia nigra evokes predominantly excitatory unit responses in that brain region[9], while local application of SP to the substantia nigra accelerates dopamine release[7] and turnover[15,31] in the striatum and causes contraversive turning behavior[15]. Furthermore, infusion of an antiserum against SP into the substantia nigra decreases striatal dopamine release[6].

Little information is available concerning possible effects of striatal dopamine on SP neurons. Hong et al.[14] found that chronic treatment with haloperidol reduced SP concentrations in the substantia nigra, and suggested that this reflected increased activity of SP striatonigral neurons because an inhibitory influence had been blocked. In a previous communication, we reported that the administration of D-amphetamine reduced SP levels in the rat striatum[26]. We suggested that this reduction, which was maximal 2–4 h after amphetamine administration, might have been mediated by activation of dopaminergic receptors; however, no direct evidence was available to show that striatal dopamine was necessary for the effect. The present report further characterizes the amphetamine effect and shows its dependence on adequate striatal dopamine levels.

METHODS

Animals

Male Sprague–Dawley rats weighing 150–200 g were used in all experiments (Charles River Laboratories, Wilmington, Mass.). Prior to experimentation, the animals were maintained for several days under 12 : 12 h light: dark cycle, having free access to standard rat chow (Ralston–Purina Rat Chow) and tap water.

Drugs

The following drugs and equivalent volumes of their diluents were injected intraperitoneally: D-amphetamine sulfate in 0.9% saline (Sigma, St. Louis, Mo.), methylphenidate (Ritalin) in 0.9% saline (Ciba–Geigy, Summit, N.J.), α-methyl tyrosine methyl ester in 0.9% saline (Aldrich, Milwaukee, Wisc.), haloperidol in 50 mM citric acid (McNeil Laboratories, Fort Washington, Pa.), cycloheximide in 0.9% saline (Sigma). Intracerebral injections of 6-hydroxydopamine·HBr (6-OHDA; Sigma) were made in 0.9% saline containing 0.1% ascorbic acid as an antioxidant.

Stereotaxic procedure

6-OHDA was injected stereotaxically into substantia nigra of anesthetized rats (pentabarbital; Nembutal, 50 mg/kg i.p.) placed in a Kopf stereotaxic apparatus (Model 900). The coordinates for the intranigral injections were: AP, 2.2 mm; ML, 1.8 mm; DV, —2.4 mm (all given as mm away from the stereotaxic zero point)[19]. The 6-OHDA was injected in vols. of 2 μl (4 μg/μl as the free base) over a 2 min period using a 10 μl Hamilton syringe with a 27-gauge cannula. The cannula remained in position for 3 min after injection to allow adequate diffusion of the neurotoxin.

Assays

Tissue preparation. After decapitation, brains were rapidly removed and placed on an ice-chilled glass plate. Brain regions were dissected free-hand from coronal slices that were approximated by the following anterior–posterior planes of Konig and Klippel[19]: substantia nigra (4–5 mg wet weight), 1600–2600 μm; hypothalamus (30–35 mg wet weight), 2600–7200 μm; and head of striatum (25–30 mg wet weight), 7200–9600 μm. In the case of the striatum, care was taken not to include globus pallidus; substantia nigral tissue included both zona compacta and zona reticulata. Immediately after dissection, tissues were quickly frozen on dry-ice and stored at —70 °C until processing. To prepare tissues for the SP radioimmunoassay, samples were homogenized thoroughly in 2 N acetic acid (about 15 vols.) using a small teflon pestle; homogenates were then spun for 15 min in an Eppendorf centrifuge, and aliquots of the clear supernatant were lyophilized. If dopamine was to be measured in the same sample as SP, portions of the 2 N acetic acid extract could be used. Otherwise, for assay of striatal dopamine, the tissues were homogenized in about 15 vols. of 0.1 M perchloric acid containing 0.4 mM sodium bisulfite and centrifuged. Appropriate aliquots were taken for assay.

Substance P radioimmunoassay. The details of this assay[25] are described briefly here. The rabbit antibody was developed against SP conjugated to bovine thyroglobulin. The antiserum was used at a final dilution of 1 : 200,000 to give, in the absence of SP, a maximum binding of trace SP ([^{125}I]8-tyrosine SP) of 30–40%. (Many peptides have been tested against the antibody: the only one to exhibit significant cross-reactivity has been the structurally related amphibian peptide, physaelamine, which was about 1/200th as immunoreactive[25].) Standard curves for SP generally exhibited 50% displacement at 8–12 fmoles and could reliably detect 2–4 fmoles of SP immunoreactivity.

Dopamine assay. The striatal concentration of dopamine was assayed by high performance liquid chromatography (HPLC; cation exchange) coupled to electrochemical detection, using a slight modification of the method described by Keller et al.[18]. Samples were prepared similarly using batch adsorption to alumina[18]; however, the column conditions were somewhat altered as follows: (i) column; 2 mm × 50 cm glass column (Rainin) using Waters Corasil CX strong cation exchange resin (30–40 μm particles); (ii) mobile phase, citrate–phosphate buffer (0.025 M citric acid + 0.05 M sodium phosphate mixed to achieve pH 6.3); (iii) column pressure and flow rate, 300 psi at 0.5 ml/min; and (iv) electrode potential, +0.5 V. The electrochemical detector (model LC 2A) and flow cell were purchased from Bioanalytical Systems (West Lafayette, Ind.), and a Milton Roy Minipump was utilized to deliver the mobile phase. The retention times for internal standard (dihydroxybenzylamine) and dopamine were 6 and 9 min, respectively; the recoveries for the two amines were 50–60% for dihydroxybenzylamine and 40–50% for dopamine. (Although this assay schema is very reliable and relatively fast for dopamine analysis, it may not be adequate for the routine assay of norepinephrine, since norepinephrine elutes close to the solvent front.)

Measurement of in vivo protein synthesis. The rate of in vivo incorporation of

[^{3}H]lysine into protein was measured as described by Roel et al.[27]. Thirty min before decapitation, the rats received, while under light ether anaesthesia, an intraventricular injection of 1.5 μCi [^{3}H]lysine (4,5-[^{3}H]lysine, New England Nuclear, Boston, Mass.) in 20 μl 0.01 M Tris buffer, pH 7.0. After decapitation, striata and the rest of the brain were homogenized in water, and aliquots were taken for measurement of total radioactivity and trichloracetic acid (TCA)-insoluble radioactivity[27]. The incorporation of tritiated lysine into protein was represented by the ratio TCA precipitable radioactivity/total tissue radioactivity. Total tissue protein was assayed by the method of Lowry et al.[20].

RESULTS

Administration of D-amphetamine sulfate (10 mg/kg i.p.) significantly reduced the concentration of SP immunoreactivity in the striatum 2 h after injection by about 30% (Table I). No reductions in SP were seen in the substantia nigra or hypothalamus (Table I), nor were they seen within a single experiment in the septum or the interpeduncular nucleus. Treatment of rats at room temperature with this relatively high dose (10 mg/kg) of D-amphetamine sulfate generally increased rectal temperature by 2–3 °C after 1 h. This increase was not the cause of the amphetamine-induced reduction in striatal SP, inasmuch as striatal SP concentrations were unaffected in rats whose core temperatures were increased to an equivalent amount by being placed in a warm environment (38 °C). The reduction in striatal SP levels after D-amphetamine treatment was dose-dependent in the range of 2.5–10 mg/kg (Table II). The administration of methylphenidate (Ritalin; 50 or 100 mg/kg), a drug which, like amphetamine, releases dopamine in vivo from nerve terminals[3,21], also reduced striatal SP levels significantly 2 h after injection (Table III).

In an attempt to amplify the reductions in striatal SP after D-amphetamine or to reduce SP levels in the substantia nigra, we gave some rats frequent, repeated doses of D-amphetamine. In each of two experiments, groups of 6–8 animals were killed 2 h after receiving either a single dose of D-amphetamine (10 mg/kg) or the last of 6 similar

TABLE I

Effect of amphetamine administration on regional concentrations of substance P in rat brain

Rats were injected intraperitoneally with either D-amphetamine sulfate (10 mg/kg) or its diluent (0.9% saline) and killed 2 h later. Data are given as group means ± S.E.M. Figures in parentheses refer to group n.

Treatment	*Substance P concentration*		
	Striatum (pmol/10 mg tissue)	*Substantia nigra (pmol/mg protein)*	*Hypothalamus (pmol/10 mg tissue)*
Control	1.37 ± 0.06 (12)	6.11 ± 0.14 (12)	2.46 ± 0.06 (6)
Amphetamine	0.97 ± 0.05* (12)	6.54 ± 0.65 (12)	2.61 ± 0.04 (6)

* Differs from controls, $P < 0.01$ (Student's t-test).

TABLE II

Effects of various doses of D-*amphetamine on substance P concentrations in rat striatum*

Rats were sacrificed 2 h after intraperitoneal administration of D-amphetamine sulfate or its diluent (0.9% saline). Values are group means ± S.E.M. Figures in parentheses refer to group n.

Dose (mg/kg)	*Substance P concentration (pmol/10 mg tissue)*
0	1.24 ± 0.06 (12)
2.5	1.19 ± 0.04 (12)
5	1.07 ± 0.05 (6)
10	0.98 ± 0.04* (12)

* Differs from zero dose group, $P < 0.05$ (ANOVA, Newman–Keuls test).

TABLE III

Effect of methylphenidate administration on striatal substance P concentrations

Rats were injected intraperitoneally with methylphenidate or an equal volume of 0.9% saline and killed 2 h later. Values are group means ± S.E.M. (n = 6).

Dose (mg/kg)	*Substance P concentration (pmol/10 mg tissue)*
0	1.14 ± 0.06
50	0.94 ± 0.07*
100	0.84 ± 0.08*

* Differs from zero dose group, $P < 0.05$ (ANOVA, Newman–Keuls test).

doses given 5 h apart. The 5 h interval between injections was chosen because it had been determined previously[26] that a single injection of D-amphetamine lowers striatal SP levels maximally between 2 and 4 h after injection, and that SP returns to normal after 8 h. While a single injection of D-amphetamine reduced striatal SP concentrations, its repeated administration failed to do so (Table IV). No changes were observed in substantia nigra SP levels after either a single dose or frequent, repeated doses of D-amphetamine (Table IV).

Pretreatment of rats with haloperidol (1, 3 or 10 mg/kg i.p.), a potent dopamine receptor antagonist[5], had no effect on striatal SP levels, but blocked the amphetamine-induced fall at all 3 doses tested (Table V). Similarly, pretreatment with the tyrosine hydroxylase inhibitor, α-methyl tyrosine[29], failed to modify striatal SP concentrations but blocked the amphetamine effect (Table VI). The administration of α-methyl tyrosine depleted striatal dopamine by 70%, while amphetamine alone slightly increased dopamine levels (Tables VI and VII). Unilateral lesions of the substantia nigra, produced by injecting 8 μg of 6-OHDA one week earlier, had no effect on striatal SP levels, but reduced striatal dopamine concentrations by about 75% (Table VII). D-Amphetamine continued to reduce SP in the intact striatum (contra-

TABLE IV

Substance P concentrations in striata and substantia nigra after single or repeated doses of D-amphetamine

Rats were injected intraperitoneally one or more times (6 doses, given every 5 h) with saline or D-amphetamine sulfate (10 mg/kg) and killed 2 h after the last injection. Values are group means ± S.E.M. for two experiments (n = 10–12).

Treatment	*Striatum (pmol/10 mg tissue)*	*Substantia nigra (pmol/10 mg protein)*
Acute saline	1.52 ± 0.06	9.88 ± 0.43
Acute amphetamine	1.16 ± 0.05*	9.82 ± 0.66
Repeated saline	1.50 ± 0.06	10.83 ± 0.41
Repeated amphetamine	1.40 ± 0.06	10.54 ± 0.43

* Differs from other 3 groups, $P < 0.01$ (ANOVA, Newman–Keuls test).

TABLE V

Blockade by haloperidol of D-amphetamine-induced reduction in striatal substance P levels

Rats were first injected intraperitoneally with either 1, 3 or 10 mg/kg haloperidol or its vehicle (50 mM citric acid). Thirty min later they received D-amphetamine sulfate (10 mg/kg) or its diluent (0.9% saline); they were sacrificed 2 h later. Values are group means ± S.E.M. for two experiments (n = 10–12)

Treatment	*Substance P concentration (pmol/10 mg tissue)*
Control	1.50 ± 0.06
Amphetamine	1.14 ± 0.03*
10 mg/kg haloperidol	1.43 ± 0.06
1 mg/kg haloperidol + amphetamine	1.60 ± 0.04
3 mg/kg haloperidol + amphetamine	1.42 ± 0.13
10 mg/kg haloperidol + amphetamine	1.49 ± 0.08

* Differs from all other groups, $P < 0.01$ (ANOVA, Newman–Keuls test).

TABLE VI

Effect of α-methyl tyrosine on reduction of striatal substance P concentration after amphetamine injection

Rats were injected with either 0.9% saline or α-methyl tyrosine methyl ester (225 mg/kg i.p.) and 2 h later were challenged with 0.9% saline or D-amphetamine sulfate (10 mg/kg i.p.). Three hours after the amphetamine injection the animals were killed. Values are group means ± S.E.M. (n = 6).

Treatment	*Substance P concentration (pmol/10 mg tissue)*	*Dopamine concentration (pmol/mg tissue)*
Control	1.46 ± 0.06	75.5 ± 3.7
Amphetamine	1.21 ± 0.04*	81.4 ± 4.8
α-Methyl tyrosine	1.60 ± 0.08	20.7 ± 1.6**
α-Methyl tyrosine + amphetamine	1.49 ± 0.05	24.6 ± 0.8**

* Differs from all other groups, $P < 0.01$ (ANOVA, Newman–Keuls test).
** Differs from control and amphetamine-treated groups, $P < 0.01$ (ANOVA, Newman–Keuls test).

TABLE VII

Effect of unilateral lesions of the nigrostriatal tract by 6-OHDA on the reduction of striatal substance P concentration by D-*amphetamine*

Rats were injected with 8 μg 6-OHDA unilaterally into the substantia nigra. One week later they were challenged with D-amphetamine sulfate (10 mg/kg i.p.) and killed 2 h later. Data are represented as group means ± S.E.M. The values for substance P were combined from 2 identical experiments (n = 14) and the dopamine values were taken from a single study (n = 8).

Treatment	*Substance P concentration (pmol/10 mg tissue)*	*Dopamine concentration (pmol/mg tissue)*
Innervated control	1.78 ± 0.09	73.0 ± 2.4
Innervated amphetamine	1.34 ± 0.06*	85.3 ± 3.7***
Lesioned control	1.74 ± 0.08	19.7 ± 5.5**
Lesioned amphetamine	1.59 ± 0.04	15.1 ± 3.2**

* Differs from other 3 groups, $P < 0.05$ (ANOVA, Newman–Keuls test).
** Differs from values for both innervated striata, $P < 0.01$ (ANOVA, Newman–Keuls test).
*** Differs from respective control group, $P < 0.05$ (ANOVA, Newman–Keuls test).

lateral to the lesion), but had no significant effect on SP levels in the lesioned side (Table VII).

In our previous communication[26], we suggested that one mechanism by which D-amphetamine might have reduced striatal SP levels would be by suppressing its biosynthesis; amphetamine has been shown to disaggregate brain polysomes and to inhibit the in vivo incorporation of amino acids into protein[22,27]. We tested this possibility by examining the effect on striatal SP of another drug, cycloheximide, which is known to inhibit brain protein synthesis[1]. Two hours after rats received a

TABLE VIII

Effect of acute inhibition of in vivo brain protein synthesis on the concentration of substance P in the striatum and substantia nigra

Rats were injected intraperitoneally with 0.9% saline, cycloheximide (120 mg/kg) or D-amphetamine sulfate (10 mg/kg). After 90 min, the rats received an intraventricular injection of [^{3}H]lysine (1.5 μCi in 20 μl of 0.01 M Tris buffer, pH 7); they were decapitated 30 min later. Data are given as group means ± S.E.M. (n = 8); the per cent tritiated lysine incorporation equals TCA-precipitated radioactivity, divided by whole tissue radioactivity × 100.

Treatment	*Substance P concentration*		*% [^{3}H]lysine incorporation*	
	Striatum (pmol/10 mg tissue)	*Substantia nigra (pmol/mg protein)*	*Striatum*	*Rest of brain*
Control	1.37 ± 0.12	8.51 ± 0.47	38.8 ± 2.1	36.1 ± 1.0
Cycloheximide	1.51 ± 0.12	8.28 ± 0.41	0.1 ± 0.1**	0.5 ± 0.1**
Amphetamine	1.05 ± 0.07*	8.39 ± 0.51	29.8 ± 2.1	29.0 ± 3.0

* Differs from other two groups, $P < 0.05$ (ANOVA, Newman–Keuls test).
** Differs from other two groups, $P < 0.01$ (ANOVA, Newman–Keuls test).

large dose of cycloheximide (120 mg/kg i.p.), striatal SP levels were unchanged, even though the drug had almost totally inhibited in vivo protein synthesis (Table VIII). (In additional studies, we found that this dose of cycloheximide almost completely stopped in vivo brain protein synthesis throughout the 10–180 min period following its administration.) Conversely, D-amphetamine reduced striatal SP levels, as usual, 2 h after injection, while causing only a moderate (20–23 %) inhibition of protein synthesis (Table VIII).

DISCUSSION

These studies have shown that the acute administration of D-amphetamine sulfate lowers the concentration of immunoreactive SP in the striatum in a dose-dependent manner (Table II). Although the reduction is relatively modest (20–30 %), it is rapid and highly consistent. The reduction indicates that striatal SP stores are labile and can respond to pharmacologic manipulations in relatively short periods of time. That the reduction in SP was observed only in the striatum, and not in substantia nigra, suggests that amphetamine preferentially affects perikarya of SP neurons; that no reduction was seen in the hypothalamus suggests that amphetamine does not act directly on SP neurons, but acts trans-synaptically.

The striatum has one of the highest dopamine concentrations in the brain, which is reflected in its input of nigrostriatal neurons[10]. This in itself raises the possibility that D-amphetamine, which is known to enhance dopamine release[2], reduces striatal SP levels by enhancing dopaminergic transmission. This possibility is further supported by our observations that methylphenidate also lowers SP concentrations in the rat striatum 2 h after injection (Table III). The doses of methylphenidate used here (50 or 100 mg/kg i.p.) have been shown previously to increase markedly brain dihydroxyphenylacetic acid and homovanillic acid at 2 h[3], and thus probably to enhance dopamine release[21]. Various manipulations which interfered with dopaminergic neurotransmission antagonized the amphetamine effect. These manipulations included pretreatment with the receptor-blocking agent, haloperidol, or the synthesis-inhibitor, α-methyl tyrosine (Tables V and VI), or destruction of the dopaminergic nigrostriatal tract by 6-OHDA (Table VII). Although none of these experimental manipulations alone produced significant changes in striatal SP levels, their ability to block amphetamine's effect is compatible with the possibility that striatal dopamine release normally participates in the regulation of neuronal SP concentrations in this brain region. Our observations do not, however, rule out the possibility that a polysynaptic mechanism involving the nigrostriatal tract mediates amphetamine's effect on striatal SP.

In contrast to what was seen after a single dose of D-amphetamine, repeated doses given over a 27 h period did not lower SP concentrations (Table IV); nigral levels were unaffected after either dosage regimen. Thus, it appears that a rapid tolerance develops to amphetamine's effects on striatal SP. The mechanism of such a tolerance is unknown. We have not explored the effects of chronic daily administration of D-amphetamine on striatal or nigral SP. It is quite possible that tolerance to the

effects of the drug would also develop in this circumstance. Hong et al.[14] demonstrated that chronic treatment with haloperidol reduced SP levels in the substantia nigra. This could have resulted from an enhanced release of the peptide from nerve terminals of the striatonigral tract. It would be difficult to compare the results of the present study with those of Hong et al.[14] until more detailed information becomes available on the precise cellular loci at which the two drugs act.

If amphetamine does reduce striatal SP levels by enhancing dopaminergic transmission, one might then expect that administration of apomorphine, a dopamine agonist[28], would mimic the amphetamine effect. We have, however, failed to detect consistently significant changes in SP 2 h after systemic injection of various doses of apomorphine (2.5, 5 or 10 mg/kg; unpublished observations). This discrepancy may reflect differential agonist and antagonist actions of apomorphine on subclasses of striatal dopamine receptors[8,17].

The precise cellular mechanism by which amphetamine reduces striatal SP concentrations is unknown, but may involve several possibilities. For example, amphetamine could act via dopamine to release SP from striatal neurons; this could be followed by SP's degradation to non-immunoreactive compounds. (We observe that SP can be released from striatal minces, in vitro, by high potassium concentrations (30–60 mM) in the presence of calcium — unpublished observations). However, if SP is released from nerve terminals after amphetamine, it might have been expected that SP levels would decrease in the substantia nigra instead of in the striatum; at no time (15–480 min) after amphetamine injection was this observed[26]. It is of course conceivable that amphetamine releases SP from striatal cell bodies or dendrites; however, dendritic release of SP has not yet been demonstrated. If a portion of striatal SP is stored within small intrinsic neurons (i.e. besides the portion in perikarya of the striatonigral tract), amphetamine might lower striatal SP by releasing it from terminals of such intrinsic neurons.

Amphetamine might reduce striatal SP by interfering with its synthesis. Most likely, its site of action would be on the enzymatic post-translational modification of a hypothetical SP precursor molecule, inasmuch as the near-total inhibition of protein synthesis per se by cycloheximide fails to alter SP levels, in contrast to amphetamine (Table VIII). The failure of SP levels to fall after this degree of inhibition could mean that SP neurons contain so much of a hypothetical precursor that its levels do not become limiting for synthesis of the SP fragment even when production of the precursor has been suppressed for 2 h. It is also possible that SP levels fail to fall after cycloheximide injection because this drug inhibits the syntheses of both SP and an enzyme that metabolizes the peptide. This mechanism would require that this hypothetical enzyme have an extraordinarily rapid half-life for its activity to disappear so soon after its synthesis is suppressed.

ACKNOWLEDGEMENTS

D.J.P. was supported in part by predoctoral fellowships from the Whittaker Health Sciences Fund and the National Institute of Mental Health. These studies were

supported in part by a grant from the United States Public Health Service (AM-14228).

We thank Ciba-Geigy for giving us a supply of Ritalin, and Dr. Susan Leeman for supplying us with a substance P antiserum and valuable advice.

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