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EFFECTS OF SUBSTANCE P INJECTED INTO THE SUBSTANTIA NIGRA

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1 Behavioural and biochemical effects of substance P (SP, 1 to 10 μg) administered in a small volume to discrete areas of the rat's brain were studied by means of a refined microinjection technique.

2 SP injected unilaterally into the zona reticulata of the substantia nigra elicited dose-dependent contraversive circling and an increase in dopamine turnover in the ipsilateral striatum. SP applied to the zona compacta or zona lateralis, or to the medial lemniscus, evoked ipsiversive turning with a fall in dopamine turnover and a rise in 5-hydroxytryptamine (5-HT) turnover in the corresponding striatum.

3 In both cases the onset of turning was immediate, reached a peak at about 5 min and lasted for 10 min. Both types of behaviour were blocked by haloperidol and exaggerated by nialamide.

4 Unilateral injections of SP given into the crus cerebri, zona incerta, caudate nucleus, putamen or globus pallidus did not modify the animal's behaviour.

5 In rats pretreated with apomorphine or amphetamine, SP induced contraversive circling which was followed by locomotion in the opposite direction.

6 Turning responses to a second dose of SP were diminished at 3 h and reproducible at 24 h after the first injection.

7 Bacitracin (50 ng) injected into the zona reticulata caused ipsiversive turning. Larger intranigral doses of bacitracin (10 μg), as with intracisternal SP (10 μg), evoked 'barrel rotation'.

8 No changes in the free concentrations of aspartate, glutamate, γ-aminobutyric acid, glycine or alanine were detected in any brain region following an intracisternal injection of 10 μg SP, although glutamine levels were elevated throughout the brain 30 to 60 min later.

Introduction

There is now considerable evidence for the existence of an excitatory striato-nigral pathway which utilizes substance P (SP) as its neurotransmitter (for review see Mroz, Brownstein & Leeman, 1977). It is quite possible this pathway functions to counterbalance the parallel inhibitory γ-aminobutyric acid (GABA)-mediated input to the substantia nigra (SN), thereby enabling the activity of the nigrostriatal dopaminergic system to be finely controlled (Dray & Straughan, 1976). Thus SP injected unilaterally into the SN has been shown to increase dopamine cell activity ipsilaterally, as evidenced by a heightened impulse flow in the ascending nigrostriatal dopaminergic neurones (Davies & Dray, 1976), by an increase in dopamine turnover in the corresponding striatum (Chéramy, Nieoullon, Michelot & Glowinski, 1977; James & Starr, 1977), and by a compulsion in freely moving animals to turn towards the uninjected side (James & Starr, 1977; Olpe & Koella, 1977). However, what is not certain, is whether these effects reflect a direct and specific excitant action of SP on the dopaminergic cells, or whether SP is also capable of modifying the activities of other fibre groups within the basal ganglia.

In the present study we have attempted to clarify this point by investigating the biochemical and behavioural effects of administering SP into discrete areas of the basal ganglia and their environs by a refined microinjection technique (James & Starr, 1978). The results show that SP is only active when introduced into the neighbourhood of the SN, and that it can either accentuate or attenuate dopaminergic cell activity depending on the exact placement of the SP injection within this nucleus.

Methods

Male Wistar albino rats (Tuck), 200 to 250 g, were lightly anaesthetized with halothane (1% in O_2) and injected stereotaxically with a 1 μl Hamilton syringe.

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Drug solutions (0.1 μl) were expelled slowly over 5 min in order to achieve maximal initial localization of the drug within a sphere having an approximate volume of 0.04 mm^3 (see James & Starr, 1978). Upon recovery from the anaesthetic (approximately 2 min) the rats were placed in a rectangular box (0.3 m wide, 0.55 m long) and observed for 10 min for signs of postural bias, or for preferred locomotion to one side, in which case the frequency of rotation was scored. Afterwards the animals were killed and their brains quickly removed and frozen in a solid CO_2/methanol mixture. They were kept in this state until assayed, when they were allowed to thaw sufficiently to enable the corpora striata to be dissected out. These were then weighed on a torsion balance and assayed fluorimetrically, by established methods, for dopamine (Shellenberger & Gordon, 1971), homovanillic acid (HVA, Murphy, Robinson & Sharman, 1969), 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA, Curzon & Green, 1970). Results are expressed as μg/g wet wt.

Rats receiving multiple injections into the nigra were first implanted unilaterally with a stainless steel guide cannula (24 gauge) fixed to the skull with acrylic cement. A recovery period of 1 week was allowed to elapse before injections were made into the nigra via a 32 gauge needle inserted into the guide cannula to a predetermined depth. Injections (1 μl) were expelled slowly over a period of 2 min into conscious animals with an Agla micrometer syringe. Injection sites were always verified histologically.

In another set of experiments rats were anaesthetized lightly with ether and SP (10 μg) injected in a volume of 10 μl through the cisterna magna into the cerebrospinal fluid. On regaining consciousness the animals were first observed for signs of abnormal behaviour and then killed and the brains quickly removed and frozen as above. These were then dissected into seven major regions according to the method of Glowinski & Iversen (1966) and the nigras identified and removed. Small fractions of each area were weighed and their free amino acid content analysed radiometrically by the dansylation method (Snodgrass & Iversen, 1973). Amino acid concentrations were expressed as μmol/g wet wt. of tissue.

Materials

[^{14}C]-amino acids for use as internal standards and [^{3}H]-dansyl chloride were obtained from the Radiochemical Centre, Amersham. Routine laboratory chemicals were of analytical quality, except for the solvents employed for liquid scintillation spectrometry, which were of scintillator grade. Substance P was purchased from the Protein Research Laboratories, Osaka, Japan. (+)-Amphetamine was obtained from Koch Light, apomorphine from MacFarlan Smith and haloperidol from Searle Laboratories (Morpeth). Nialamide was kindly donated by Pfizer.

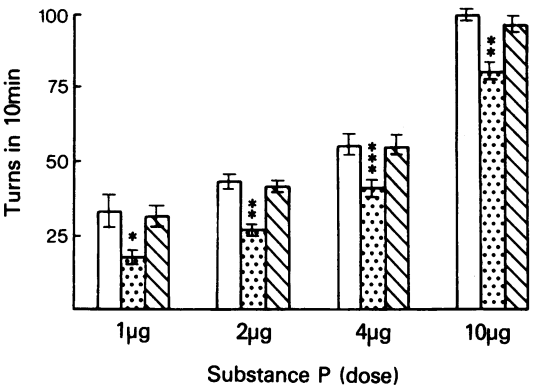

Figure 1 Dose-related contralateral turning induced by microinjections of substance P(SP) into the substantia nigra zona reticulata. Rats received an initial dose of SP (open columns) followed either 3 h (stippled columns) or 24 h later (hatched columns) by a second injection. Results are the means of 6 experiments; vertical lines show s.e. mean. $*P < 0.02$, $**P < 0.01$, $***P < 0.001$ versus initial dose SP (Student's t-test).

Results

Behavioural responses to substance P

SP (1 to 10 μg) injected unilaterally into the rostral, central or caudal region of the substantia nigra zona reticulata (SNR) elicited a dose-dependent contraversive circling response (Figure 1), characterized by slow and intermittent unidirectional turning on the spot with animals frequently pausing to sniff or groom, or to make occasional exploratory movements. Turning began immediately upon recovery from the anaesthetic, reached a peak frequency at 5 min and lasted for 10 min (Figure 2a). In contrast, untreated and saline (0.9% w/v NaCl solution)-injected control rats were never observed to circle in this fashion, although they sometimes moved through 360° whilst exploring the periphery of the box. However, the net 10 min turning score for a group of 10 control animals was invariably zero.

When the injection of SP into the SNR was preceded 15 min earlier by the intraperitoneal administration of apomorphine (1 mg/kg), a typical contraversive turning response was evoked which was then followed by locomotion in the opposite direction (Figure 2b). This ipsiversive rotation continued to increase in intensity over the ensuing 10 min, reaching a maximum 3.8 ± 0.2 turns/min ($n = 6$, $P < 0.001$

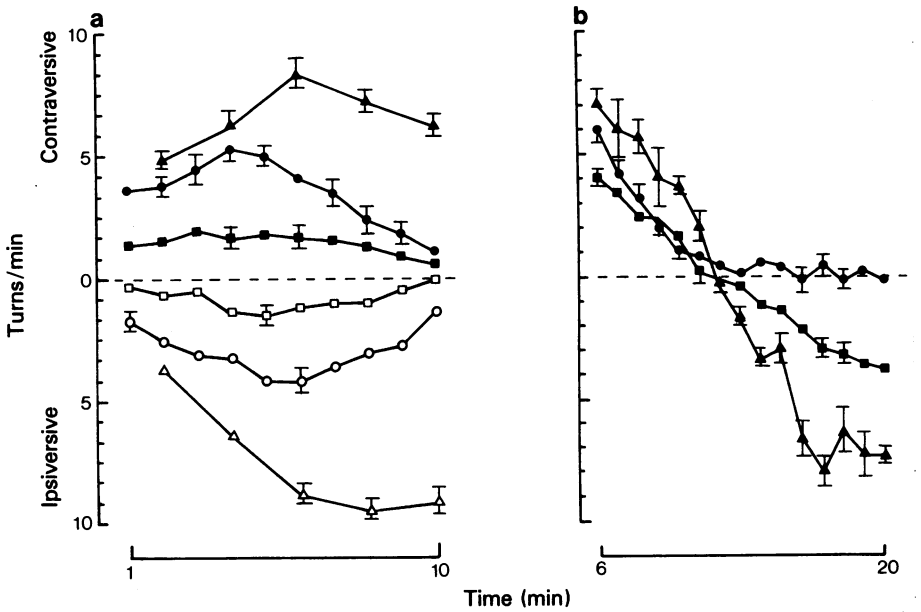

Figure 2 (a) Time course of contraversive (closed symbols) and ipsiversive turning (open symbols) elicited by injections of 1 μg substance P in control rats (circles) and in rats pretreated with haloperidol (squares, 0.5 mg/kg, i.p., 15 min earlier) or nialamide (triangles, 100 mg/kg 90 min earlier). Control and haloperidol-treated animals were scored every min, whilst those receiving nialamide were scored every 2 minutes. Results are the means of 6 experiments, vertical lines show s.e. means. Different groups of rats were used for each drug treatment. (b) Biphasic turning responses elicited by injections of 1 μg substance P into the SNR of rats pretreated 15 min. earlier with apomorphine (squares, 1 mg/kg, i.p., $n = 6$) or amphetamine (triangles, 2.5 mg/kg, $n = 5$). Control rats (circles, $n = 6$) were pretreated with saline vehicle. Results are the means; vertical lines show s.e. mean.

versus saline controls), at which point the animals were killed. A similar reversal of the turning response to SP was produced by amphetamine pretreatment (2.5 mg/kg i.p. 15 min beforehand), reaching 7.3 ± 0.3 turns/min ($n = 5$, $P < 0.001$) 20 min after SP injection (Figure 2b). When either of these drugs was administered to saline-injected control rats, the animals became visibly hyperactive but showed no signs of preferential movement to one side.

A second intranigral application of SP (1 to 10 μg) to conscious rats, given 3 h later into the same site as the first, proved significantly less effective as a behavioural stimulus (Figure 1). However, the turning response to SP was not diminished, when the first injection was 1 μl intranigral saline, or when the two doses of peptide were administered 24 h apart (Figure 1).

Contraversive rotation produced by SP was significantly attenuated by pretreating rats 15 min earlier with haloperidol (0.5 mg/kg i.p.; $P < 0.001$, Figure 2a), without altering the time course of the turning response. This dose of haloperidol had no discernible effect on the behaviour of saline-injected animals. Nialamide (100 mg/kg i.p. given 90 min earlier) significantly increased both the number of turns ($P < 0.001$) and the duration of turning ($P < 0.01$) elicited by SP (Figure 2a). On the the other hand, saline-treated control animals receiving nialamide became noticeably more active than untreated rats, but did not rotate.

Interestingly, unilateral injections of 1 to 10 μg SP (but not saline) into the substantia nigra zona compacta (SNC) or pars lateralis (SNL), or into the medial lemniscus, caused the animals to rotate ipsiversively (Figure 2a). This behaviour was also inhibited by haloperidol and facilitated by nialamide (dosages as above; Figure 2a), whilst intraperitoneal pretreatment with apomorphine (1 mg/kg) or amphetamine (2.5 mg/kg), administered 15 min earlier, had no effect on the response to SP. Similar injections of SP into the globus pallidus, caudate nucleus, putamen, crus cerebri or zona incerta failed to induce any form of rotatory behaviour (Figure 3).

Of the 31 rats administered 10 μg SP intracisternally (i.c.), 24 exhibited 'barrel rotation' (see Cohn & Cohn, 1975). On recovering from the anaesthetic (2 to 3 min) these rats immediately began to 'rotate' or 'roll' on the spot about the longitudinal head-to-tail axis of the body, the direction of rotation varying between animals. These movements ranged in frequency from 6 to 18 rotations/min and usually lasted 5 to 10 min; the animals remained hyperexcitable to

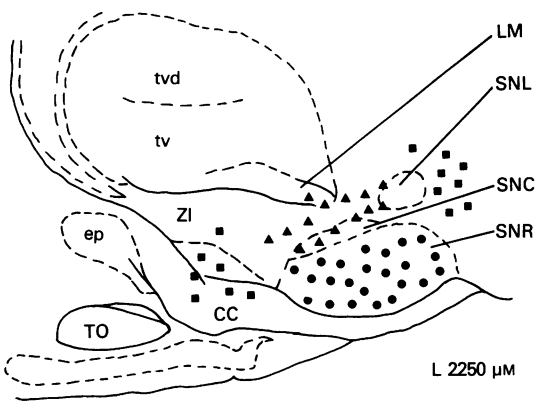

Figure 3 Schematic diagram showing the sites of injection of substance P (SP) made into the ventral midbrain region of the rat's brain. Microinjections of SP (1 to 10 μg, 0.1 μl over 5 min) elicited either contralateral (●) or ipsilateral (▲) turning, or had no effect (■) on locomotor activity. LM = medial lemniscus, SNL = substantia nigra zona lateralis, SNC = substantia nigra zona compacta, SNR = substantia nigra zona reticulata, ZI = zona incerta, CC = crus cerebri, tvd = dorsoventral thalamus, tv = ventral thalamus, ep = endopenduncular nucleus, TO = optic tract.

auditory and tactile stimuli for a further 60 min without bilateral asymmetry.

Bacitracin injected unilaterally into the SNR in small doses (50 ng) produced ipsiversive turning (48.1 ± 5.1 turns in 20 min, $n = 6$, $P < 0.005$ versus saline controls). At higher dose levels (10 μg) bacitracin induced 'barrel rolling'; this behaviour continued intermittently for about 60 min and then reverted to the more typical ipsiversive circling for another hour before the animals eventually became quiescent. Recovery from the drug appeared to be complete on the following day.

Biochemical changes with substance P

It can be seen from Table 1 that control injections of saline made directly into, or in the immediate vicinity of the SNR or SNC did not alter the ipsilateral striatal concentrations of dopamine or 5-HT, or of their principal deaminated acidic metabolites, HVA and 5-HIAA respectively. Nor were there any significant bilateral differences in the striatal levels of these four compounds in non-injected, saline-injected or sham-injected controls (not shown). One μg of SP delivered into the SNR by microinjection (0.1 μl) significantly lowered the dopamine content of the ipsilateral striatum (12.0%, $P < 0.01$, Table 1), whilst raising the corresponding level of HVA (53.7%, $P < 0.001$). No differences in the striatal concentrations of 5-HT or 5-HIAA were evident in these animals (Table 1).

In contrast, animals moving ipsiversively in response to SP injected into the SNC, SNL or medial lemniscus, revealed a drop in striatal dopamine turnover on the side of the injection; ipsilateral striatal dopamine levels rose by 18.2% ($P < 0.02$) and HVA levels fell by 27.0% ($P < 0.01$, Table 1). In these rats striatal 5-HT levels remained unchanged, although there was a significant increase in the ipsilateral striatal 5-HIAA content (50.0%, $P < 0.001$).

In other experiments, the free levels of aspartic and glutamic acid, glycine, GABA, glutamine and alanine were determined in various brain regions following the intracisternal administration of 10 μg SP, in an attempt to verify an earlier observation that SP causes a widespread elevation in brain glycine levels

Table 1 Effects of intranigral injection of substance P (SP) on turning behaviour and on the striatal levels of dopamine, homovanillic acid (HVA), 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA)

Direction of rotation		*Striatal concentration (μg/g wet wt.)*			
		Dopamine	*HVA*	*5-HT*	*5-HIAA*
None (controls)	L	1.47 (6)	0.45 (10)	1.04 (6)	0.38 (6)
	R	1.48 (6)	0.41 (10)	0.98 (6)	0.37 (6)
Ipsiversive	L	1.32 (7)	0.48 (10)	1.06 (7)	0.34 (9)
	R	1.56 (7)*	0.35 (10)**	1.03 (7)	0.51 (9)***
Contraversive	L	1.84 (7)	0.41 (12)	0.91 (6)	0.41 (6)
	R	1.62 (7)**	0.63 (12)***	0.93 (6)	0.34 (6)

Rats were injected stereotaxically in the right SN with 1 μg SP in a volume of 0.1 μl under light halothane anaesthesia. Upon recovery the rats were placed in a rectangular box and their locomotor behaviour observed for 10 min. The animals were then killed and their brains quickly dissected and assayed for the compounds shown. Results are the mean of the number of experiments shown in parentheses. All s.e. mean lay in the range ± 6–10%. L = left hand side, R = right hand (injected) side.
* $P < 0.02$; ** $P < 0.01$; *** $P < 0.001$ versus opposite side (paired t test).

(Stern, Catovic, & Stern, 1974). The results for SN and corpus striatum are presented in Table 2. At no time during the post-injection period (0.5 to 24 h) did SP significantly alter the free concentrations of aspartic acid, glutamic acid, glycine, GABA or alanine in the SN, striatum, cerebellum, hypothalamus, hippocampus, cerebral cortex or medulla/pons. The only differences found to be statistically significant were the small widespread increases in brain glutamine detected 30 to 60 min after administering SP, averaging as much as 32% in the SN ($P < 0.01$) and as little as 12% in the striatum ($P < 0.05$).

Discussion

Using a refined microinjection technique we have shown that a unilateral injection of SP made discretely into the SNR increases striatal dopamine turnover ipsilaterally and stimulates rats to turn contraversively, whilst SP applied unilaterally to the SNC, SNL or medial lemniscus has the opposite effect. Both behaviours are attenuated by haloperidol and accentuated by nialamide and are therefore consistent with SP exciting or inhibiting dopaminergic cell activity respectively on the injected side. The stimulant action of SP on nigral dopaminergic neurones concurs with earlier electrophysiological (Davies & Dray, 1976) and biochemical observations (Duffy, Wong & Powell, 1975), whilst the diminished turning response elicited by repeated intranigral injections of SP agrees well with the reported refractoriness of nigral SP receptors to prolonged iontophoretic application of the peptide (Davies & Dray, 1976). These and earlier immunocytochemical data, which indicate the existence of specific SP-containing afferents to the SN (Kanazawa, Emson & Cuello, 1977), are considered to offer strong supporting evidence for the proposed role of SP as an excitatory neurotransmitter in this nucleus.

A plausible explanation of SP-evoked ipsiversive circling, which has not been described previously, is that SP is also capable of exciting hypothetical inhibitory interneurones. Since these have been conjectured to impinge upon the cell bodies or dendrites of the nigrostriatal dopaminergic cells (Dray & Straughan, 1976), it follows that increased firing of these intrinsic fibres will strengthen their inhibitory influence on the dopaminergic system in that hemisphere and thereby initiate ipsiversive locomotion. The accompaniment of this type of behaviour by an apparent rise in striatal 5-HT turnover is also noteworthy and is in agreement with the findings of other workers who injected large doses of synthetic SP (60 μg) into the lateral ventricles (Magnusson, Carlsson, Fisher, Chang & Folkers, 1976). However, whilst 5-hydroxytryptaminergic (5-HTergic) fibres may pass close to or terminate in the SN, they are not known to originate there (Dahlstrom & Fuxe, 1964; Dray, Gonye, Oakley & Tanner, 1976). Consequently, the mechanisms underlying this action of SP require clarification, but

Table 2 Effects of intracisternal substance P (SP) on free amino acid levels in rat substantia nigra and corpus striatum

Time after SP (*h*)	*Amino acid concentration (μmol/g wet wt.)*					
	Aspartic acid	*Glutamic acid*	*Glutamine*	*Glycine*	*Alanine*	*GABA*
Substantia nigra						
0 (controls)	2.53	4.28	2.36	1.23	0.30	6.89
0.5	3.02	4.24	3.12**	1.21	0.35	6.84
1	2.98	4.70	2.11**	0.87	0.31	6.89
2	3.12	4.72	2.17	1.06	0.43	6.82
24	3.06	4.87	2.78	1.23	0.36	6.88
Corpus striatum						
0 (controls)	2.01	5.53	3.75	0.46	0.57	1.66
0.5	2.03	5.31	4.29*	0.53	0.68	1.86
1	2.11	5.61	4.30*	0.45	0.69	1.68
2	2.63	5.46	3.57	0.57	0.69	1.45
24	2.67	5.69	3.43	0.50	0.66	1.59

Rats were injected intracisternally with 10 μg SP in a volume of 10 μl under light ether anaesthesia. They were then killed at the various times indicated and free amino acid levels determined by dansylation. Results are the mean of eight experiments. All s.e. mean lay in the range ± 6–14%.
* $P < 0.05$; * $P < 0.01$ versus controls (Student's t test).

a direct effect of SP on 5-HTergic fibres cannot be excluded.

That both forms of rotation induced by SP were typically slow and short-lived could be due to the small number of neurones influenced by the microinjection, to the rapid inactivation of the peptide by catabolism (Benuck & Marks, 1975) or lipid binding (Lembeck, 1977), or to receptor desensitization (Davies & Dray, 1976). Alternatively other neuronal circuits may rapidly compensate for any bilateral imbalance in the activity of the nigrostriatal dopaminergic system. On the other hand, a deleterious effect of the anaesthetic is unlikely, since closely similar results were obtained with SP administered to the nigra in conscious rats (see also Olpe & Koella, 1977).

From earlier work it is apparent that drug-induced rotational behaviour can vary according to the level of impulse traffic in the ascending dopaminergic pathways. It is interesting, therefore, that stimulating striatal dopamine receptors with apomorphine or amphetamine introduced an additional phase of ipsiversive turning to the animals' movements, following a typical 10 min period of SP-induced contraversive circling. These results suggest that striatal dopamine receptor dominance has shifted from the injected to the non-injected side of the brain, but the reasons for this are not apparent at present.

Bacitracin is an antipeptidase agent and was administered to the nigra with a view to prolonging the postsynaptic action of any 'transmitter SP' that might have been liberated endogenously. However, injections of bacitracin into the SNR stimulated rats to turn ipsiversively, which did not conform to the expected pattern of behaviour attending the accumulation of SP in this part of the SN and may therefore have been due to other pharmacological actions of the compound. The curious 'barrel rotation' observed here with bacitracin and SP has also been noticed with intranigral applications of picrotoxin and kainic acid (James & Starr, unpublished observations), and following massive intracerebroventricular injections of somatostatin (Cohn & Cohn, 1975) and SP (Magnusson *et al.*, 1976). Since this form of axial rotation was often preceded or followed by the circling motion that is characteristic of unilateral nigrostriatal dopaminergic cell activation (Ungerstedt, 1971), we believe that it may be an additional manifestation of the latter phenomenon.

We cannot corroborate the findings of Stern *et al.* (1974) that glycine is instrumental in the production of sterotyped behaviours with SP, since we failed to observe the SP-induced elevation in brain glycine levels reported by these authors. The concentrations of free alanine, aspartic acid, glutamic acid and GABA were similarly unaffected by intracisternal SP, and in our opinion the behavioural changes evoked by SP in rats are more likely to be due to an interaction of the peptide with aminergic nerves. SP-sensitive adenylate cyclase is distributed widely among various brain structures (Duffy *et al.*, 1975), so that a large intracisternal injection of SP might be expected to reach and depolarize neurones over a wide area. Consequently, the increased nitrogen clearance that would accompany such a widespread increase in nerve cell activity could account for the increase in free glutamine levels that we detected 30 to 60 min after administering SP intracisternally.

In conclusion, our present findings support the concept that SP may function as a neurotransmitter in the SN by showing that injections of the peptide into this nucleus elicit behavioural and biochemical responses closely consistent with those expected of the peptide released endogenously. Thus within the zona reticulata SP appears to stimulate dopaminergic neurones, whereas SP administered immediately dorsal to this region may indirectly suppress dopaminergic cell activity by stimulating an inhibitory interneurone.

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