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IN VIVO BIOSYNTHESIS OF [^{35}S]- AND [^{3}H]SUBSTANCE P IN THE STRIATUM OF THE RAT AND THEIR AXONAL TRANSPORT TO THE SUBSTANTIA NIGRA[1]

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Abstract

The biosynthesis and axonal transport of the neuropeptide substance P (SP) in the striatonigral tract of the rat was examined using an *in vivo* radiolabeling of the rostral corpus striatum and a series of high performance liquid chromatography (HPLC) steps for the purification of radiolabeled SP. The corpus striatum of unrestrained rats was continuously infused via indwelling cannulae for 16 hr with [^{35}S]methionine or a mix of [^{3}H]leucine and [^{3}H]proline. Radiolabeled SP was acid extracted from discrete regions of this striatonigral SP projection—corpus striatum (SP-immunopositive cell bodies), ansa lenticularis (striatonigral SP axons), and substantia nigra (striatonigral SP terminals)—and was purified to a constant specific activity by sequential HPLC. The radiochemical purity of SP was verified by chemical derivative formation (SP-Met^{11}-sulfoxide) and further HPLC.

The *in vivo* labeling procedure resulted in a high level of incorporation of the amino acids into tissue protein and peptide pools. [^{35}S]SP and [^{3}H]SP were positively identified in all three regions of this peptidergic projection. The amount of [^{35}S]SP harvested in each region accounted for 0.0015%, 0.003%, and 0.071% of the total tissue ^{35}S present in the striatum, striatonigral fibers, and substantia nigra, respectively. The amount of [^{3}H]SP harvested in each region accounted for 0.0025%, 0.011%, and 0.27% of the total tissue ^{3}H present in the three regions, respectively. The amount of radiolabeled SP in the striatonigral regions for both isotopic infusion studies was highly correlated with the immunoassayable SP content in those regions, suggesting rapid equilibration of *de novo* biosynthesized SP with striatonigral tissue pools of SP.

Tritium autoradiography of the striatonigral projection after ^{3}H-amino acid infusion provided further support for the specificity of the radiolabeling procedures. Heavily labeled fibers were seen leaving the striatal infusion site caudally, forming a distinct fiber bundle. This bundle projected caudoventrally and formed a dense terminal plexus primarily within the reticulata portion of the substantia nigra.

These results demonstrate that SP biosynthesis in the corpus striatum and its transport to the substantia nigra can be studied in discrete striatonigral regions obtained from individual unrestrained rats. This preparation should allow for studies on the dynamics of SP biosynthesis, axonal transport, and turnover in the striatonigral projection.

Substance P (SP) is an undecapeptide originally detected in extracts of brain and intestine in 1931 on the basis of its muscle-contracting and hypotensive properties (von Euler and Gaddum, 1931). It was subsequently isolated in pure form from bovine hypothalamic tissue extracts and identified as Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (Chang and Leeman, 1970; Chang et al., 1971). SP has a wide but unequal distribution in the mammalian central nervous system (CNS), as determined by bioassay, radioimmunoassay, and immunohistochemistry (Pernow, 1953; Lembeck and Zetler, 1962; Brownstein et al., 1976; Cuello and Kanazawa,

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1978; Ljungdahl et al., 1978; Nicoll et al., 1980). High concentrations of SP are found in the dorsal horn of the spinal cord, in the nucleus of the descending tract of the trigeminal nerve, and in various subcortical regions of the brain, including the substantia nigra, amygdala, and hypothalamus.

Within the substantia nigra, SP is largely found in fibers that form a dense terminal plexus in the pars reticulata. The majority of these SP fibers appear to originate from SP-containing neuronal cell bodies in the rostral portion of the corpus striatum (Mroz et al., 1976; Hong et al., 1977; Brownstein et al., 1977; Kanazawa et al., 1977; Jessell et al., 1978). Within the substantia nigra pars compacta and pars reticulata, SP is present in both myelinated and nonmyelinated fibers and is contained within large granular vesicles, (Duffy et al., 1975; Cuello et al., 1977) in axon terminals which form primarily axodendritic synapses (DiFiglia et al., 1981). SP has been shown to be released from nigral synaptosomes (Schenker et al., 1976) or slices (Jessell, 1978) in a calcium-dependent and tetrodotoxin-sensitive manner. It has also been reported that microiontophoretically applied SP exerts an excitatory effect on nigral cells (Davies and Dray, 1976; Walker et al., 1976) and that the striatonigral SP projection neurons may exert facilitatory influences on nigrostriatal dopaminergic neurons (Cheramy et al., 1978; Waldmeier et al., 1978). Therefore, it appears that SP may play a major regulatory role within the striatonigral system as a neurotransmitter or neuromodulatory substance.

Of primary importance to a satisfactory understanding of striatonigral regulation by SP is the elucidation of its metabolic pathway and an understanding of the regulation of its biosynthesis and axonal transport. Toward this end, we present some of our initial observations on the *in vivo* incorporation of radiolabeled amino acids into the SP molecule, after continuous infusion of [^{35}S]methionine or [^{3}H]leucine and [^{3}H]proline in the rostral striatum, the area of SP-positive neuronal perikarya. By sequentially purifying SP from discrete regions of the striatonigral projection by high performance liquid chromatography (HPLC), newly synthesized SP was detected in the rostral striatum, in the region of the ansa lenticularis (area of SP axons), and in the substantia nigra (area of SP nerve terminals). The radiolabeling protocol used (amino acid delivery to discrete brain regions) should be of general use in the study of CNS protein and peptide biosynthesis and their axonal transport. Thus, SP biosynthesis, axonal transport, and turnover may be examined in the striatonigral projection in individual unrestrained rats using these amino acid labeling and SP purification strategies. A preliminary report of these studies has appeared (Krause et al., 1982b).

Materials and Methods

Synthetic SP, SP-free acid, Tyr^{8}-SP, and Gln-Gln-Phe-Gly-Leu-Met-NH_2 were obtained from Peninsula Laboratories (San Carlos, CA). Dr. Shmaryahu Blumberg generously provided the following partial sequences of SP: Boc-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, pGlu-Phe-Phe-Gly-Leu-Met-NH_2, Phe-Phe-Gly-Leu-Met-NH_2, Phe-Gly-Leu-Met-NH_2, Gly-Leu-Met-NH_2, Boc-Leu-Met-NH_2, and Boc-Phe-Phe-Gly-Leu-Met-Gly. These SP fragments were used as HPLC and radioimmunoassay standards. HPLC grade acetonitrile and methanol were obtained from Burdick and Jackson Laboratories (Muskegon, MI). The following radiolabeled amino acids were purchased from New England Nuclear Corporation: L-[3,4,5-^{3}H (*N*)]leucine (110 Ci/mmol), L-[2,3,4,5-^{3}H]proline (160 Ci/mmol), and L[^{35}S]methionine (1175 Ci/mmol). All other chemicals were the highest purity available (Sigma Chemical Co., St. Louis, MO, or Fisher Scientific Co., Pittsburgh, PA).

Experimental paradigm. The general sequence of steps involved in the *in vivo* infusion of radiolabeled amino acids into the striatum and for the purification of SP from the microdissected striatum, striatonigral axons, and substantia nigra is presented in Figure 1.

Experimental animals and cannulation procedures. Male rats (60 to 90 days old, 175 to 200 gm) of the Sprague-Dawley strain were used. They were housed in our animal facilities for 1 week under controlled conditions of light (14 hr on, 10 hr off) and temperature (21 to 23°C) prior to experimentation. *Ad libitum* access to food and water was provided.

For cannulation, rats were anesthetized with ether and were placed in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA). A sagittal scalp incision was made above the cannulation site, and a burr hole was made in the calvarium to the level of the dura. The rats were unilaterally implanted with two 28 gauge stainless steel cannulae (Small Parts, Inc., Miami, FL) in the right corpus striatum (coordinates A +1.5 mm, L 2.0 mm and 3.0 mm, and V 5.0, mm, using bregma as reference). The implanted cannulae were affixed to the calvarium by using ivory grip cement (LD. Caulk Co., Milford, CA), and a machine screw (Small Parts, Inc.) was attached to the skull approximately 6 mm posterior to the implanted cannulae. A piece of Tygon tubing was centered around the implanted cannulae for protection and was affixed with grip cement to the cannulae cement support. The skin around the implanted cannulae was sutured, and the rat was allowed to recover for 24 to 36 hr prior to experimentation. The radiolabeled amino acid solutions were lyophilized and resuspended (at a concentration of 20 μCi/μl) in 0.05 M sodium phosphate, pH 7.4, containing 0.15 M sodium chloride.

Prior to amino acid infusion, the rats were lightly anesthetized with ether and were placed in the stereotaxic instrument. Each of the two infusion cannulae was connected to approximately 25 mm of polyethylene tubing which was filled with the ^{35}S- or ^{3}H-amino acid solution(s). This polyethylene tubing was connected to each cannula, and 2 μl of amino acid solution were delivered to the infusion site with the aid of a microliter syringe (Hamilton Co., Reno, NV) over a 10-min period. The polyethylene tubing was then connected to the outlet tubing of an osmotic minipump (Alzet 2001, Alza Corp., Palo Alto, CA). The osmotic minipumps were filled with 0.02% Coomassie brilliant blue in distilled water and were pre-incubated in 0.9% saline for 12 hr at 37°C prior to use. The minipumps were subcutaneously implanted in the area of the lower thoracic spinal cord. The polyethylene tubing was coiled and affixed to the rat by clear

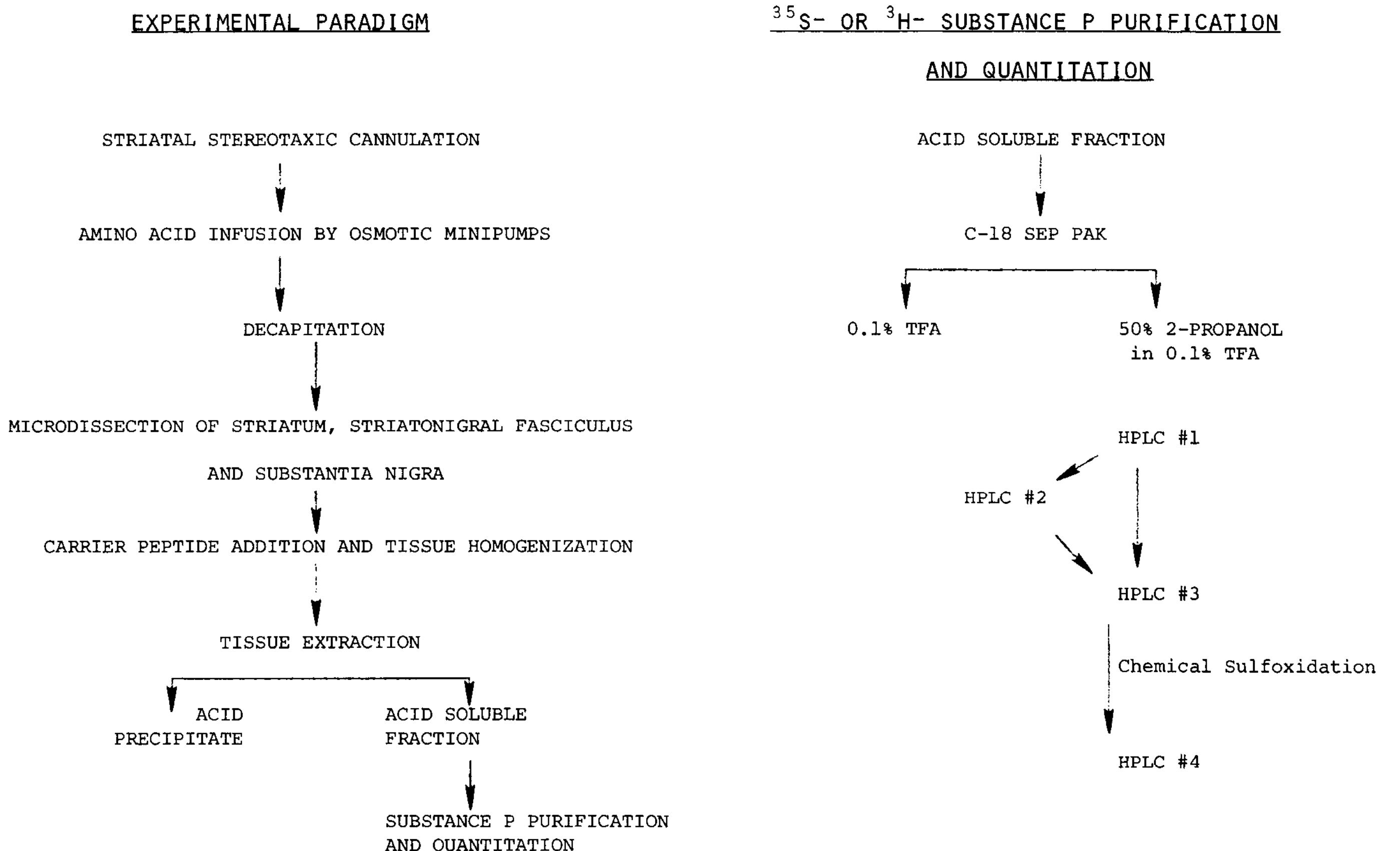

Figure 1. Experimental strategy for the study of the *in vivo* biosynthesis of striatonigral [^{35}S]- or [^{3}H]substance P. The *left portion* of the figure depicts the various stages of the experimental paradigm. The *right portion* of the figure lists the steps involved in the purification and quantitation of radiolabeled SP.

adhesive tape which was anchored to the Tygon tubing around the implanted cannulae (for protection). The rat was then taken out of the stereotaxic instrument and was allowed to recover from anesthesia. Each radiolabeled amino acid solution continuously flowed to the infusion site at a rate of 20 μCi/μl/hr/cannula. The flow of the amino acid solution was easily monitored by observing the displacement of the amino acid solution by the Coomassie dye (separated by 2 μl of air). At the end of the labeling period (16 hr) the rats were lightly anesthetized, killed by decapitation, and the cannulae were removed. The brain was removed and was frozen on dry ice.

Tissue dissections and extraction. The ipsilateral corpus striatum, the ipsilateral striatonigral fasciculus (ansa lenticularis), and the ipsilateral substantia nigra were separately dissected from frozen coronal brain sections as depicted in Figure 2 using the following rostral-caudal coordinates (reference point is bregma): striatum, +2.5 mm to +0.5 mm; striatonigral fibers, + 0.5 mm to −2.5 mm; and substantia nigra, −2.5 mm to −5.0 mm. The frozen tissue samples were homogenized in glass microhomogenizers (Micro-Metric Instrument Co., Cleveland OH) containing 2.0 M acetic acid, 2.5% β-mercaptoethanol, 50 μg of carrier SP and lug carrier amino acid(s). The acid homogenates were transferred to flint glass tubes, boiled for 5 min to inactivate proteases, and stored at −80°C prior to processing.

The striatal, striatonigral fiber, and substantia nigra samples were processed for quantitation of total tissue radioactivity (^{35}S or ^{3}H), radioactive trichloracetic acid (TCA; 10%, w/v)-precipitable protein, 10% TCA-soluble radioactivity, and [^{35}S]- or [^{3}H]SP. The 2.0 M acetic acid homogenate was made 10% with respect to TCA by addition of a 100% TCA solution and was centrifuged at 2560 × *g* in a Beckman J-6B refrigerated centrifuge. The resulting pellet was re-extracted with 10% TCA in 2.0 M acetic acid containing 2.5% β-mercaptoethanol and centrifuged as above. The supernatants were combined and were passed over a C-18 Sep-Pak cartridge (Waters, Inc., Milford, MA).

The cartridges were rinsed with 5 ml of 0.1% (v/v) trifluoroacetic acid (TFA), and the peptide-containing fraction was eluted with 3 ml of 50% (v/v) 1-propanol in 0.1% TFA. The propanol eluate from the C-18 cartridge was lyophilized prior to HPLC.

High performance liquid chromatography. Solutions for liquid chromatography were prepared with deionized glass distilled water prefiltered with a Milli Q water purification system (Millipore Corp., Bedford, MA). Solvent mixtures for HPLC were prepared with HPLC-grade acetonitrile which was filtered through an FH

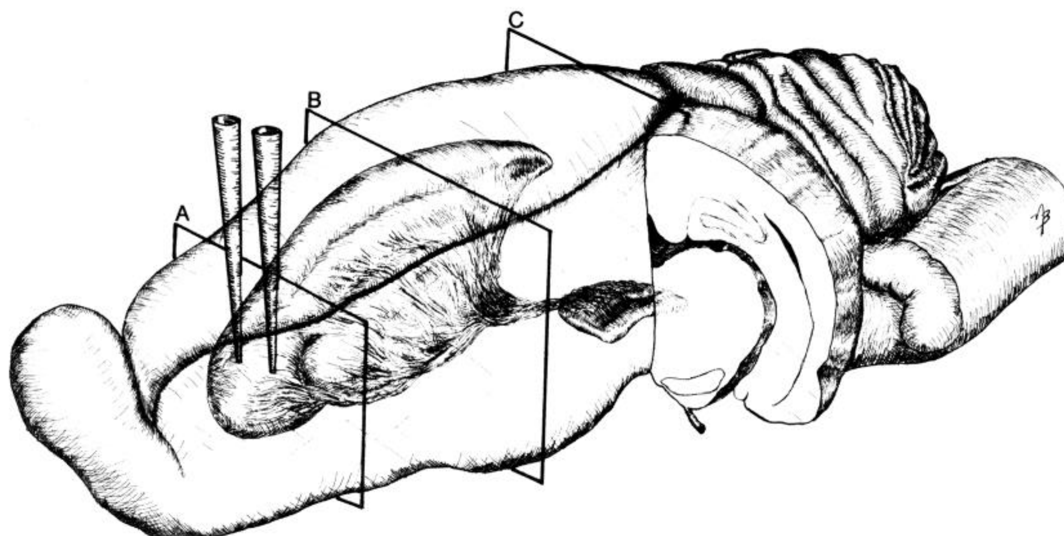

Figure 2. Schematic illustration of the rat striatonigral projection and the cannulation scheme for the study of the *in vivo* biosynthesis and transport of substance P. As depicted, the right corpus striatum is cannulated for amino acid infusion, and the three regions of this peptidergic projection (the area of cell bodies, axons, and terminals) are separately dissected for biochemical analyses. A coronal section (*A*) is placed approximately at the caudal end of the rostral portion of the corpus striatum, the area of substance P cell bodies which project to the substantia nigra. Another coronal section (*B*) is placed approximately through the middle of the ansa lenticularis, the area of striatonigral substance P axons. A final coronal section (*C*) is placed at the caudal end of the substantia nigra. Other details are provided in the text.

Millipore filter (0.5 μm). Aqueous HPLC solutions were filtered through an HA Millipore filter (0.45 μm).

The HPLC assembly consisted of a Waters Associates liquid chromatograph equipped with a model U6K injector, two model 6000A pumps, and a model 660 programmer. Ultraviolet detection was performed using a Waters model 450 variable wavelength absorbance detector. A Hewlett-Packard (Palo Alto, CA) model 3388 integrating computer was used to quantitate peak area. Chromatography was performed using 4 mm × 30 cm reverse phase C-8 or C-18 columns (10 μm particle size; Brownlee Labs, Santa Clara, CA).

Purification of radiolabeled substance P. The lyophilized propanol eluates from the C-18 cartridges were subsequently analyzed by sequential HPLC. Prior to chromatography, the lyophilized propanol eluates were resuspended in 100 μl of 0.1% TFA and clarified by centrifugation at 5000 × g/min. The following chromatographic systems were used: (*1*) HPLC 1—chromatography on a C-8 column using an exponential acetonitrile gradient (program 3, 1 to 28% CH_3CN) in 0.1 M sodium phosphate and 0.1 M phosphoric acid, pH 2.5 (buffer A) over a 50-min period at a 1 ml/min flow rate; (*2*) HPLC 2—chromatography of SP-co-migrating material on a C-18 column using a linear acetonitrile gradient (1 to 60%) in buffer A over a 50-min period at a 1 ml/min flow rate; (*3*) HPLC 3–chromatography of SP-co-migrating material on a C-18 column using an exponential acetonitrile gradient (program 3, 1 to 28%) at a 1 ml/min flow rate; (*4*) HPLC 4—chromatography of SP sulfoxide under conditions for HPLC 1. The SP peak (visible and quantifiable at 210 nm due to the presence of carrier peptide) was collected; 10% of it was taken for specific radioactivity determination, and the remainder was lyophilized and then redissolved in 0.1% TFA for subsequent HPLC. The quantity of SP carrier recovered was determined by digital integration of the absorbance of the SP peak at 210 nm and was related by linear regression to a standard curve obtained with synthetic SP. A standard curve was determined for each HPLC system. The amount of carrier SP added, relative to the amount of acid-soluble 210-nm absorbing material, and the chromatographic separation of SP from this material and from SP proteolytic and oxidation products in these systems ensured that quantitation by absorption measured only undecapeptide SP. Similar procedures were used for the quantitation of SP-Met^{11}-sulfoxide.

Sulfoxidation of radiolabeled substance P. For all tissue samples, the radiochemical purity of the isolated radiolabeled SP was verified after specific derivative formation to SP-Met^{11}-sulfoxide (Floor and Leeman, 1980) and further HPLC. The SP-containing fraction(s) from the penultimate HPLC step (after specific radioactivity determination) was lyophilized and resuspended in a solution containing 25 μl of 12 M hydrochloric acid, 50 μl of glacial acetic acid, and 10 μl of dimethyl sulfoxide (Brot et al., 1981). The reaction tube was sealed with Parafilm (American Can Co., Greenwich, CT) and was incubated at 22°C for 30 min. This sulfoxidation reaction mixture was subjected directly to HPLC as described above. Quantitation of SP-met^{11}-sulfoxide was performed by subjecting synthetic SP to the sulfoxidation reaction described above, followed by HPLC using system 4. Under these reaction conditions, the conversion of SP to SP-Met^{11}-sulfoxide was quantitative.

^3H and ^{35}S liquid scintillation spectrometry. Aliquots of tissue extracts were taken for ^3H or ^{35}S radioactivity determinations using a Beckman liquid scintillation

counter. All radioactivity determinations were performed in a ^{3}H window channel and a total β-energy channel. Radioactivity from HPLC profiles was measured in 100-μl or 1000-μl HPLC aliquots in 10 ml of Scinti Verse (Fisher) liquid scintillant. Overall ^{3}H efficiency was 45%, and ^{35}S efficiency was 92%. Tritium radioactivity was quenched by the HPLC eluate; thus, a quench correction was used for all ^{3}H counting.

Substance P radioimmunoassay. SP radioimmunoassay was performed using a rabbit anti-SP antiserum (R-5) generously provided by Drs. J. Kessler and I. Black (Cornell Medical School). Synthetic SP served as the reference preparation, and Tyr^8-SP was used for radioiodination as described (Mroz and Leeman, 1979). The iodination reaction mixture was partially purified by Sep-Pak adsorption and elution as described above, followed by HPLC on a C-8 column using an exponential acetonitrile gradient (program 3, 1 to 23% CH_3CN) over a 50-min period. This HPLC system resolves monoiodo-Tyr^8-SP (42-min retention time) from its noniodinated, diiodinated, and sulfoxide forms. The linearity of the radioimmunoassay procedure (using log/logit regression) extended from 15 to 90% of the total binding. The sensitivity of the asssay, defined by 80% of the total binding, was from 3 to 5 pg. The SP antiserum bound 30 to 40% of the [^{125}I]Tyr-SP in the buffer control tubes at a 1:240,000 final dilution. The R-5 antiserum displayed equimolar cross-reactivity with Met^{11}-SP sulfoxide and varying degrees of cross-reactivity with amidated C-terminal sequences of SP. Unreactive peptides (<0.01%) included Phe-Gly-Leu-Met-NH_2, Gly-Leu-Met-NH_2, Arg-Pro-Lys-Pro, bombesin, neurotensin, arginine vasopressin, oxytocin, luteinizing hormone-releasing hormone, α-melanocyte-stimulating hormone, β-endorphin, cholecystokinin, angiotensin, methionine enkephalin, and leucine enkephalin.

Tritium autoradiography. Following a 16-hr infusion with ^{3}H-amino acids as described above, some rats were anesthetized and perfused transcardially with 0.75% sodium chloride in 0.1 M potassium phosphate, pH 7.4, followed by perfusion with a modified Carnoy's solution (6:1, 95% ethanol:glacial acetic acid). The cannulae were removed from the brain, and the brain was postfixed for 2 hr, dehydrated, cleared, and embedded in paraffin. Transverse sections were cut (10 μm), and every tenth section was mounted onto slides. The sections were freed of paraffin in xylene, rehydrated, coated with Kodak NTB-2 emulsion, and stored at 4°C for 3 days to 2 weeks. The slides were developed in Kodak D-19 developer at 15°C, fixed in Kodak fixer, stained with thionin, and coverslipped.

Results

In vivo biosynthesis of [^{35}S]- and [^{3}H]SP in the striatum and their transport to the substantia nigra. Rats were unilaterally cannulated in the rostral corpus striatum and 24 hr later were continuously infused with either [^{35}S]methionine or an equimolar mix of [^{3}H]leucine and [^{3}H]proline at a rate of 1 μl/hr/cannula as described under "Materials and Methods." The rats did not seem to be disturbed by the amino acid delivery system since they exhibited normal motor and grooming behavior and/or slept during most of the infusion period. Rats were decapitated after a 16-hr continuous infusion of ^{35}S- or ^{3}H-amino acid(s), and the radiolabeled SP was extracted and purified from the ipsilateral neostriatum, the striatonigral fasciculus, and the substantia nigra. A schematic illustration of the site of cannulation and the approximate regions of this anatomical projection is presented in Figure 2.

Table I summarizes the isolation and purification of [^{35}S]SP from distinct portions of the striatonigral pathway after [^{35}S]methionine infusion into the corpus striatum. The total tissue ^{35}S radioactivity in the striatonigral fiber region and in the substantia nigra was 45% and 4%, respectively, of that present in the striatal infusion region. The radioactivity present in the fiber region and in the substantia nigra presumably represents striatopallidal and striatonigral projections. In all three regions, greater than 70% of the total tissue ^{35}S is present in the form of 10% TCA-precipitable protein. Figure 3 shows the UV absorbance and ^{35}S radioactivity pattern of the HPLC 1 fractions from each of the three striatonigral regions. The corresponding radioactivity pattern represents one-tenth of the total extract recovered at this stage of purification. The retention time of carrier SP was the same in each of the three striatonigral regions and also the same as that obtained with pure standard. The radioactivity profiles of the substantia nigra and striatonigral fiber region generally showed peaks coeluting with carrier SP, while no peak was observed in striatal extracts at this purification step due to the significant levels of contaminating radioactivity. Many

TABLE I

Summary of the isolation and purification of [^{35}S]substance P from distinct striatonigral regions after [^{35}S]methionine infusion into the corpus striatum

	Region Analyzed[a]		
	Striatum	Striatonigral Fibers	Substantia Nigra
	cpm	*cpm*	*cpm*
Total tissue ^{35}S	9.2×10^7	4.1×10^7	3.3×10^6
10% TCA ppt	6.8×10^7	3.8×10^7	2.8×10^6
10% TCA-soluble ^{35}S extract	2.4×10^7	3.0×10^6	4.7×10^5
C-18 cartridge ^{35}S retentate	2.4×10^6	2.7×10^5	9.0×10^4
HPLC purification	Specific activity[b]		
1 (SP)	75,100	7,890	3,590
2 (SP)	9,150	4,180	
3 (SP)	1,210	1,230	2,640
4 (SPSO)	1,420	1,220	2,350

[a] All data are expressed as counts per minute, $n = 2$; ^{35}S counting efficiency was 88%.

[b] The specific radioactivity of [^{35}S]substance P-containing fractions is expressed as counts per minute of [^{35}S]substance P per 50 μg of carrier peptide added initially. The HPLC systems used are described in the text. The substance P (SP)-containing fractions were subjected to sulfoxidation, and the [^{35}S]substance P sulfoxide (SPSO) was quantiated in HPLC 4.

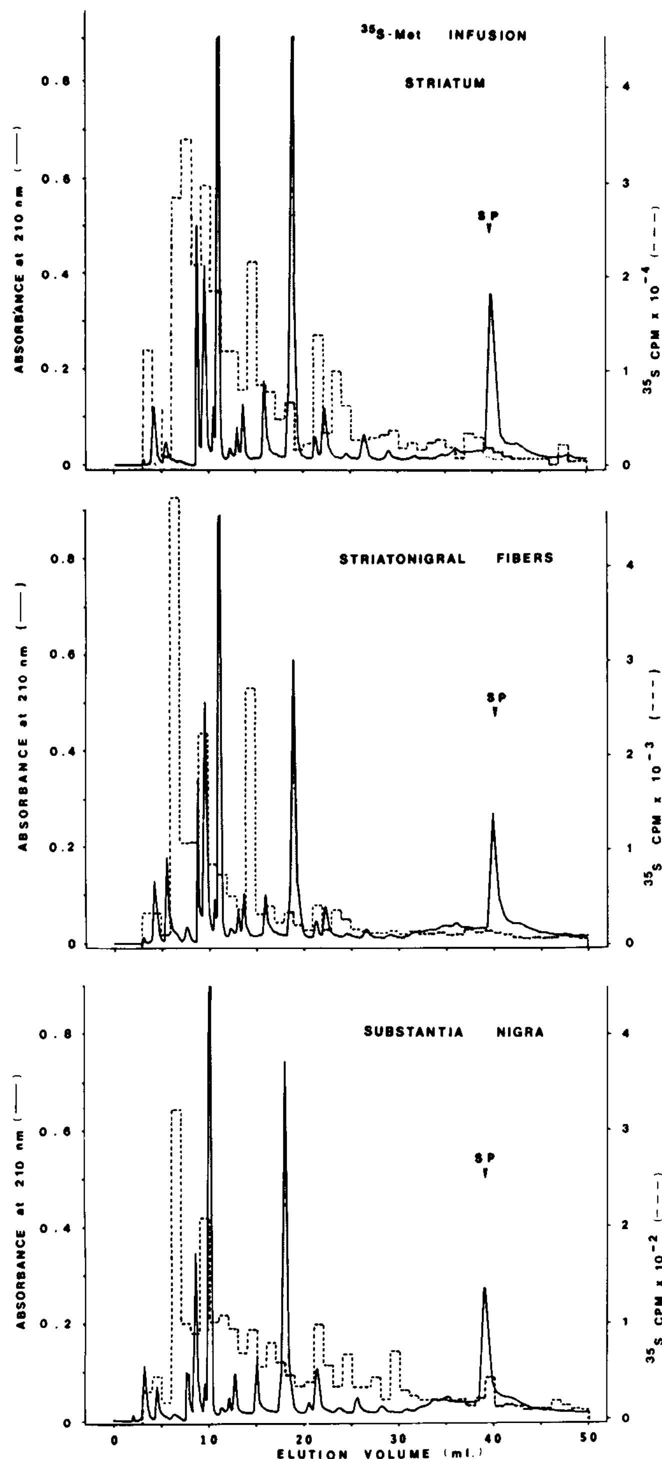

Figure 3. High performance liquid chromatography of [^{35}S] substance P from discrete striatonigral regions after continuous infusion of [^{35}S]methionine into the rostral corpus striatum. Acetic acid extracts (2.0 M) of discrete striatonigral regions were partially purified on Sep-Pak cartridges, and the Sep-Pak retentates were subjected to HPLC as described under "Materials and Methods." Ten percent of each fraction was used for liquid scintillation counting, and this was superimposed onto the absorbance profile. The remainder of the HPLC fractions co-eluting with carrier SP were lyophilized prior to further HPLC as described in the text. *SP*, substance P.

other radioactive peaks were observed with each of the three extracts.

The HPLC fractions containing carrier SP were lyophilized and resuspended prior to further HPLC. [^{35}S] SP was purified from each of the three regions by sequential HPLC to a constant specific radioactivity (Table I). Prior to the third HPLC (substantia nigra) or fourth HPLC (striatal and striatonigral fiber regions), the SP-containing fraction was converted chemically to SP-Met^{11}-sulfoxide in order to demonstrate unequivocally that the radioactivity was incorporated into the undecapeptide. The HPLC trace of the sulfoxide derivative of SP from each of the striatonigral regions is presented in Figure 4. The radiochemical purity of the isolated [^{35}S]SP (from the penultimate HPLC step) was from 89% (substantia nigra) to greater than 98% (striatal and striatonigral fiber regions) when compared to the specific radioactivity of SP-met^{11}-sulfoxide from the final HPLC step. The amount of [^{35}S]SP synthesized *de novo*, relative to the total tissue ^{35}S at the time of harvest, was 0.0015%, 0.0030%, and 0.071% for the striatum, striatonigral fasciculus, and substantia nigra, respectively.

Table II summarizes the isolation and purification of [^{3}H]SP from distinct striatonigral regions after [^{3}H] leucine and [^{3}H]proline infusion into the corpus striatum. A gradient of ^{3}H radioactivity is observed in the three regions of this projection system. The total tissue ^{3}H in the striatonigral fiber regions and substantia nigra was 21% and 2%, respectively, of that present in the striatal infusion area. From 53% (substantia nigra) to 79% (striatum) of the total tissue ^{3}H present in homogenates was in the 10% TCA-precipitable fraction. [^{3}H] SP was purified by sequential HPLC from each of the three striatonigral regions as described above for [^{35}S] SP. The radioactivity profiles from the first HPLC step (Fig. 5) showed small peaks co-eluting with carrier SP in the substantia nigra and striatonigral fiber regions, whereas no peak was observed co-eluting with carrier SP in the striatal extract chromatogram. Somewhat higher background levels of ^{3}H radioactivity were observed in each of the three regions compared to the [^{35}S]methionine labeling. Other unidentified radioactive peaks were observed in each of the three chromatograms after [^{3}H] leucine and [^{3}H]proline labeling. Many of these radiolabeled compounds had different elution properties than those observed in the [^{35}S]methionine labeling experiments. The amount of [^{3}H]SP synthesized *de novo* relative to the total tissue ^{3}H at the time of harvest was 0.0025%, 0.011%, and 0.27% for the striatum, striatonigral fasciculus, and substantia nigra, respectively.

High recovery of carrier SP was obtained at each of the HPLC steps described above, which was necessary in order to follow the specific radioactivity associated with the SP-containing fractions. Overall carrier peptide recovery from the initial extraction step up to HPLC 1 was 48.4 ± 3.6% (n = 12). Carrier peptide recoveries for HPLC 2 and HPLC 3 were 85.3 ± 2.1% (n = 8) and 88.7 ± 1.3% (n = 12). Carrier SP recovery from the chemical sulfoxidation step (using the SP fraction from the penultimate HPLC step) and subsequent HPLC was 63.2 ± 6.1% (n = 12). Thus, carrier peptide recovery for the

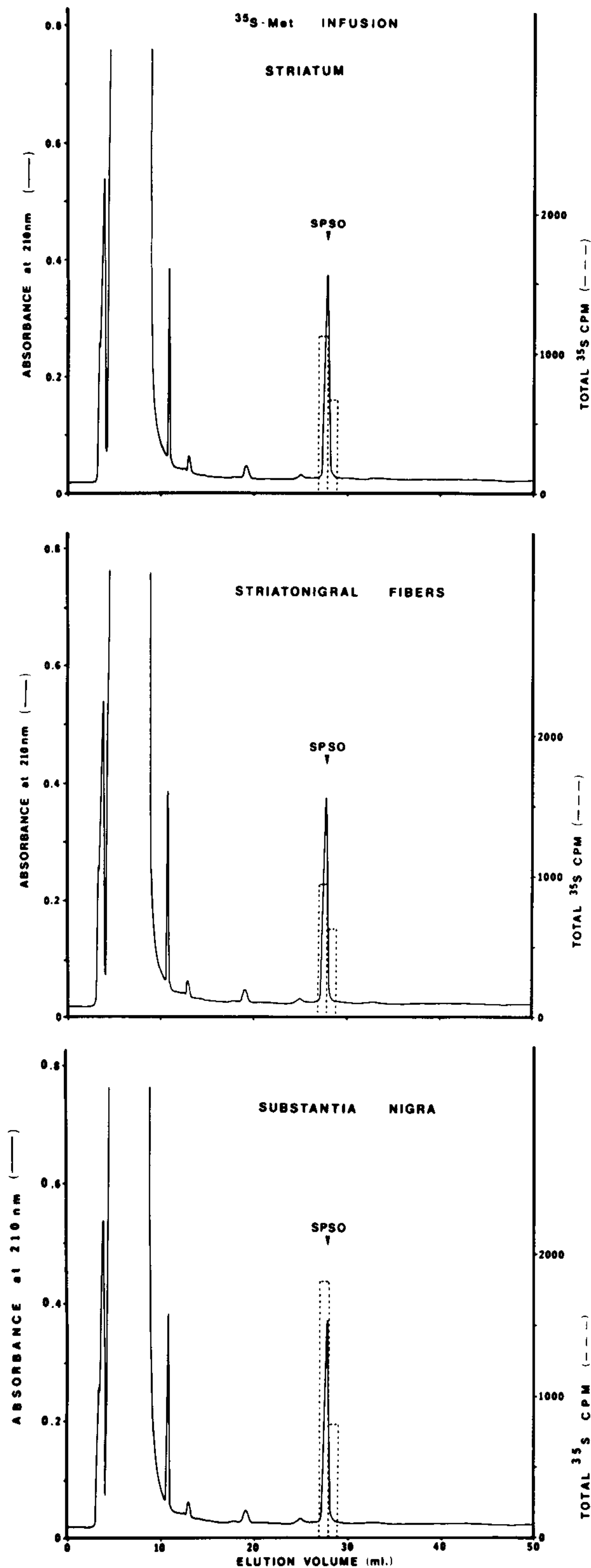

Figure 4. High performance liquid chromatography of [^{35}S] substance P sulfoxide from discrete striatonigral regions after continuous infusion of [^{35}S]methionine into the rostral corpus striatum. The SP-containing fractions(s) from the penultimate HPLC step was converted to the sulfoxide form by dimethyl sulfoxide in acid and subjected directly to HPLC as described in the text. Fifty to 100% of each HPLC fraction was used for liquid scintillation counting, and this was superimposed onto the absorbance profile (after background subtraction and correction for carrier peptide recovery). *SPSO*, substance P sulfoxide.

TABLE II

Summary of the isolation and purification of [^{3}H]substance P from distinct striatonigral regions after [^{3}H]leucine and [^{3}H]proline infusion into the corupus striatum

	Region Analyzed[a]		
	Striatum	Striatonigral Fibers	Substantia Nigra
	cpm	*cpm*	*cpm*
Total tissue ^{3}H	3.3×10^{7}	6.8×10^{6}	6.6×10^{5}
10% TCA ppt ^{3}H-protein	2.6×10^{7}	4.6×10^{6}	3.5×10^{5}
10% TCA-soluble ^{3}H extract	6.5×10^{6}	2.1×10^{6}	3.1×10^{5}
C-18 cartridge ^{3}H retentate	1.2×10^{6}	2.6×10^{5}	5.8×10^{4}
HPLC purification	Specific activity		
1 (SP)	29,100	19,900	9,060
2 (SP)	4,310	3,280	
3 (SP)	810	880	1,770
4 (SPSO)	820	780	1,750

[a] All data are expressed as counts per minute, $n = 2$. ^{3}H counting efficiency ranged from 26% to 42%, and all data were normalized to 42% efficiency. For other details, refer to Table I and the text.

entire purification was from 17 to 22% ($n = 12$). From the total amount of carrier SP added, from 25% to 35% was used in liquid scintillaton counting.

Relationship of SP content to radiolabeled SP in discrete striatonigral regions. When measured by a specific radioimmunoassay, the absolute amount of SP was approximately 2-fold greater in the substantia nigra compared to that in the striatum and striatonigral fiber regions. The SP content in the three regions on a per region basis is presented in Figure 6. The amount of SP biosynthesized *de novo* in the foregoing constant amino acid infusion experiments is highly correlated with the SP content in each of the striatonigral regions. The correlation coefficients of biosynthesized SP (counts per minute of SP per region) relative to the SP content (nanograms of of SP per region) are $r = 0.967$ and $r = 0.992$ for the ^{3}H labeling experiment and the ^{35}S labeling experiment, respectively.

Autoradiographic analysis of the striatonigral projection after striatal ^{3}H-amino acid infusion. After continuous infusion of [^{3}H]leucine and [^{3}H]proline into the rostral corpus striatum as described under "Materials and Methods," autoradiographic studies showed that a dense labeling was observed throughout the ipsilateral striatonigral projection as shown in Figure 7. Heavily labeled fibers were seen leaving the infusion site caudally, forming a distinct fiber bundle. This bundle projected caudoventrally, forming a dense terminal plexus within the reticulata portion of the substantia nigra. Most other structures adjacent to the substantia nigra showed background levels of radioactivity, as did the contralateral substantia nigra. These radiolabeled structures, particularly those in the substantia nigra, closely correspond to

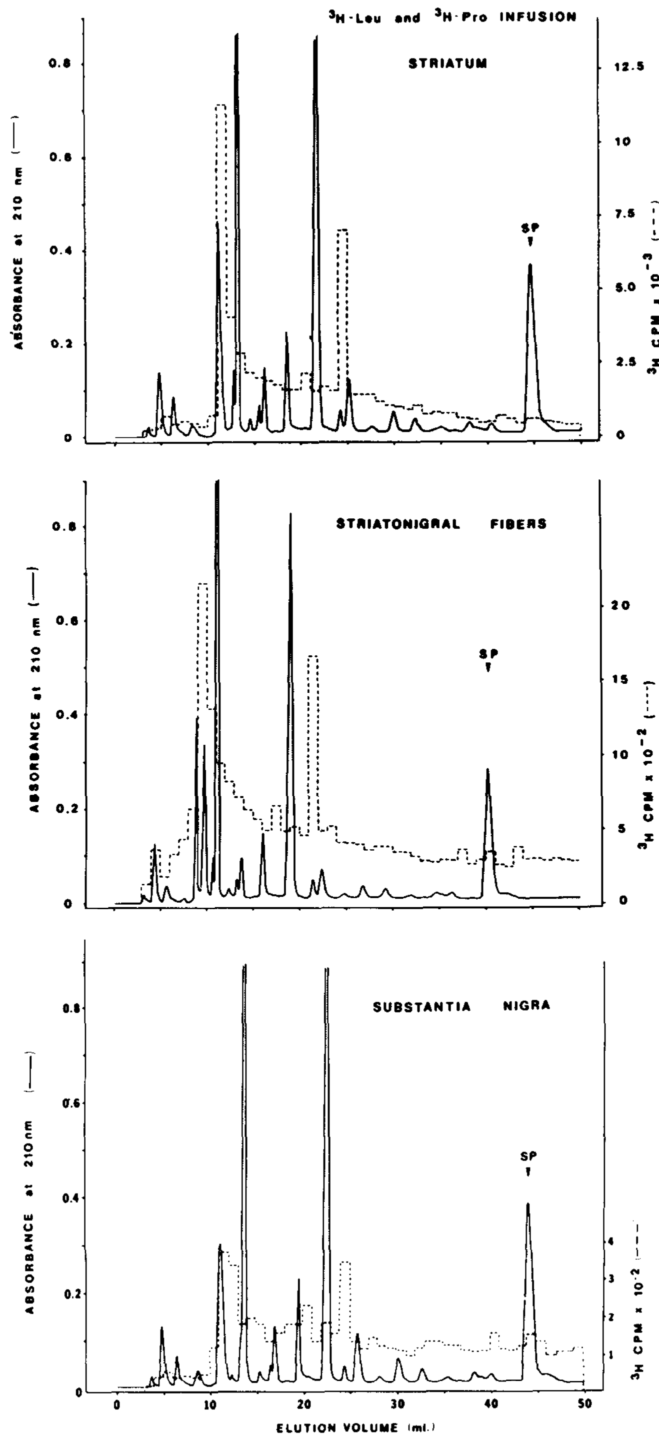

Figure 5. High performance liquid chromatography of [^{3}H] substance P from discrete striatonigral regions after continuous infusion of [^{3}H]leucine and [^{3}H]proline into the rostral corpus striatum. Details are provided in the legend to Figure 3 and in the text.

tum of rats using osmotic minipumps and a series of HPLC procedures for the rigorous purification of radiolabeled SP from discrete regions of the striatonigral projection. We have previously used similar cannulation and amino acid delivery techniques for the study of previously published SP maps in this region (Cuello and Kanazawa, 1978; Ljungdahl et al., 1978).

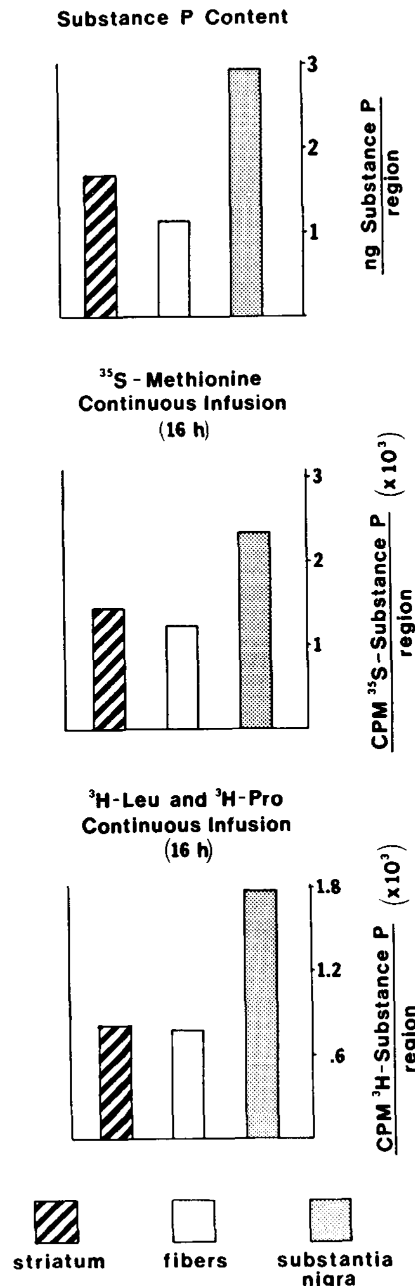

Figure 6. Comparison of substance P content with that of radiolabeled substance P in discrete striatonigral regions after continuous infusion of the rostral corpus striatum with either [^{35}S]methionine or [^{3}H]leucine and [^{3}H]proline. SP content for each striatonigral region (n = 6) was measured by radioimmunoassay as described under "Materials and Methods." Radiolabeled SP was quantitated in each of the striatonigral regions by HPLC purification and derivative formation (n = 2) as summarized in Tables I and II. In all of the histograms presented, the standard error of the mean was less than 20% of the reported mean.

Discussion

This report describes our procedures for the administration of radiolabeled amino acids into the corpus striatum of rats using osmotic minipumps and a series of HPLC procedures for the rigorous purification of radiolabeled SP from discrete regions of the striatonigral projection. We have previously used similar cannulation and amino acid delivery techniques for the study of

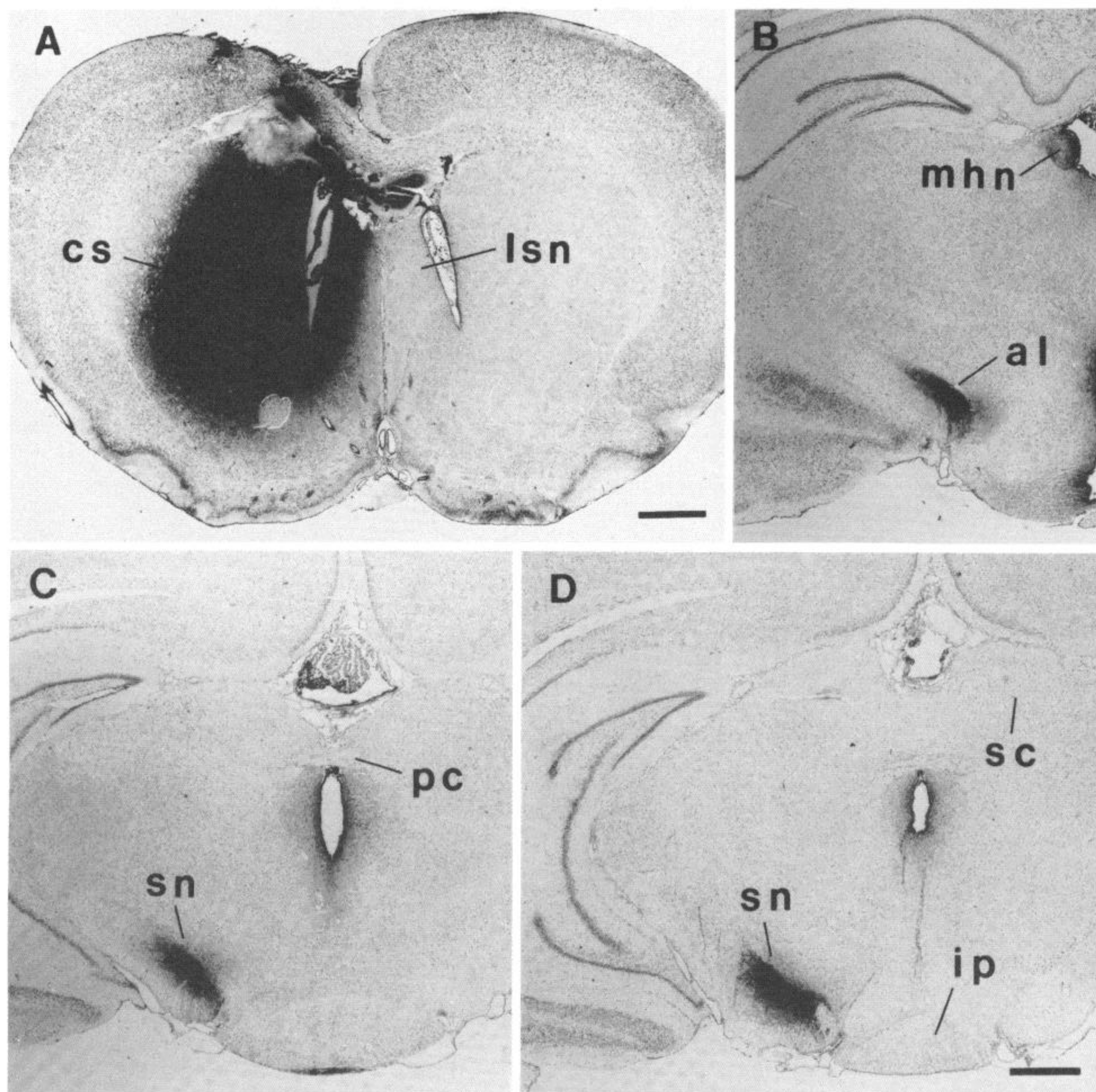

Figure 7. Tritiated amino acid autoradiography of the rat striatonigral projection after continuous infusion of [^{3}H]leucine and [^{3}H]proline into the rostral corpus striatum. Heavy silver grain label is evident in these lightfield photomicrographs in the striatum (*A*), the ansa lenticularis (*B*), and the substantia nigra (*C* and *D*). The *calibration bar* represents 1000 μm in *A* and 100 μm in *B* to *D*. *cs*, corpus striatum; *lsn*, lateral septal nucleus; *al*, ansa lenticularis; *mhn*, medial habenular nucleus; *sn*, substantia nigra; *pc*, posterior commissure; *sc*, superior colliculus; *ip*, interpeduncular nucleus.

hypothalamic LHRH biosynthesis (Krause et al., 1982a).

In the present studies, the identity of the [^{35}S]SP or [^{3}H]SP was established by purification of the undecapeptide to a constant specific radioactivity using different HPLC systems. Chemical derivatization of the isolated SP to Met^{11}-SP sulfoxide, and further HPLC unequivocally established that the radiolabeled amino acids were incorporated into the undecapeptide. Thus, strictly chemical procedures may be successfully used for the purification of SP along the trajectory of these peptidergic neurons in order to assess the dynamics of the *in vivo* biosynthesis of SP in the corpus striatum and its transport to the substantia nigra.

Immunoadsorption or bioadsorption techniques were not used in the present study. Instead, radiolabeled SP was separated from many cellular constituents by adsorption to a hydrophobic Sep-Pak cartridge. Then, peptide separation was achieved by gradient elution HPLC techniques. Within the striatum, greater than 99.99% of the ^{35}S or ^{3}H radioactivity represented contaminating radiolabeled species. Within the substantia nigra, 99.9% and 99.5% of the radioactivity present represented contaminating species in the [^{35}S]methionine and [^{3}H]leucine/[^{3}H]proline labeling, respectively. Regardless of the level of contaminating species present in each of the striatonigral regions, however, unequivocal demonstra-

tion of the level of incorporation into authentic SP was achieved by purification to homogeneity. The high recovery of carrier SP throughout the various HPLC steps was necessary to determine unequivocally the amount of isotope incorporated into the undecapeptide, particularly for the striatal and striatonigral fiber regions.

The autoradiographic analysis performed on the rat brain after [^{3}H]leucine and [^{3}H]proline labeling of the corpus striatum provides anatomical evidence which supports the specificity of the amino acid infusion paradigm. It is well established that the major efferent projections of the corpus striatum are those to the globus pallidus and to the substantia nigra (Szabo, 1962, 1967, 1970; Nauta and Mehler, 1966; Bunney and Aghajanian, 1976; Domesick, 1977; Brodal, 1981). The SP-positive cell bodies which project to the globus pallidus and to the substantia nigra have their origin in the rostral portion of the striatum (Hong et al., 1977; Brownstein et al., 1977; Jessell et al., 1978). The SP-containing axons leaving the striatum pass through the globus pallidus and enter the ansa lenticularis (Palkovits et al., 1978) as they course to the substantia nigra. The autoradiographic analysis provided herein clearly depicts this striatonigral projection, showing very dense specific labeling of the substantia nigra pars reticulata. The distribution of SP-containing terminals in the substantia nigra pars reticulata closely matches the autoradiographic distribution of silver grains that were observed in this structure. The possibility exists that some of the radioactive SP found in the region of the striatonigral fibers is contained not in axons, but in nerve terminals on entopeduncular neurons. Although some autoradiographic grains were observed in periventricular structures, this was expected since the medial-most indwelling cannula was situated approximately 0.75 mm from the lateral ventricle. The microdissection procedures used ensured that the radioactivity present in the striatonigral fiber region and substantia nigra did not include any periventricular structure.

The present studies indicate that the [^{35}S]SP and [^{3}H] SP present in each of the three striatonigral regions is highly correlated with the SP content of each region as measured by radioimmunoassay, suggesting that *de novo* biosynthesized SP equilibrates with the tissue pools of SP in this 16-hr continuous labeling time. However, the rate of SP synthesis and turnover cannot be accurately estimated until pulse-labeling experiments on SP biosynthesis in the striatonigral projection are completed (studies in progress).

Recently, two other studies have been published on the *in vivo* biosynthesis of SP in the corpus striatum and its transport to the substantia nigra. Sperk and Singer (1982) used manual injection of [^{35}S]methionine over a 4-hr period into the striatum in order to observe synthesis and colchicine-sensitive transport of SP to the substantia nigra. These workers used pooled tissues (striata or substantia nigrae) from six rats to provide evidence for synthesis and transport. [^{35}S]SP was purified by multiple chromatographic steps including gel permeation chromatography and ion exchange chromatography, with HPLC, paper electrophoresis, and immunoadsorption techniques on selected samples, necessitating long processing times. Torrens et al. (1982) implanted the striatum of rats with push-pull cannulae to study the biosynthesis of [^{35}S]SP from [^{35}S]methionine and its transport to the substantia nigra. [^{35}S]SP biosynthesis was estimated by using immunoadsorption and HPLC techniques. These studies are of interest, but it should be pointed out that the establishment of radiochemical purity is essential to quantitative studies on SP biosynthesis and dynamics, since many radiolabeled contaminants, many with properties similar to SP, are found in the tissues (Tables I and II). In addition, our studies represent an approach to striatal SP biosynthesis that can be carried out conveniently at the individual animal level with effective labeling of the protein and peptide pools.

At the present time, no reliable information exists on the chemical nature of the putative protein precursor of SP in any neuronal system, although it has been observed that the synthesis of radiolabeled SP in dorsal root ganglia explants (Harmar et al., 1981) and in the striatum *in vivo* (Torrens et al., 1982) is sensitive to the protein synthesis inhibitor cycloheximide. Our work can provide valuable information with regard to the issue of the SP precursor on two accounts. First, the results of the experiments described above define the limits on the amount of striatonigral tract SP that has its origin in the striatum. This information can be useful to those interested in defining the precursor protein to SP by recombinant DNA methods. From 0.004% ([^{35}S]methionine labeling; Table I) to 0.008% ([^{3}H]leucine and [^{3}H] proline labeling; Table II) of the total radioactivity in the striatonigral tract is present in undecapeptide SP. If the mRNA species coding for SP does not have an appreciably different half-life than other brain mRNAs, then one would expect as a minimal estimate between 0.004% and 0.008% of the mRNA species from the rat striatum to code for the SP precursor protein. Taking into account the fact that precursor proteins of other neuropeptides can be about 10-fold greater in molecular weight (Herbert and Uhler, 1982), this estimate could be as much as 10-fold higher (that is, from 0.04% to 0.08%). Secondly, using the described cannulation and radiolabeling techniques in a pulse-labeling fashion, we have observed relatively large amounts of authentic radiolabeled SP in the striatum after short time intervals (2 to 4 hr) after the start of isotopic amino acid infusion. This indicates that SP precursor processing occurs rapidly within the striatum locally. Thus, short time intervals after isotope infusion can be used in conjunction with antibodies that cross-react with SP free acid (the sequence of SP present in a putative precursor) to isolate and characterize putative precursor protein(s) to SP.

In conclusion, the present results indicate that neuropeptide biosynthesis in the CNS can be studied by using the described techniques of *in vivo* labeling of a discrete neuronal projection and a series of HPLC steps, used in conjunction with derivatization of the product peptide, to assess the *de novo* biosynthesis and axonal transport of a particular neuropeptide. These experimental and analytical procedures can be used to assess the activity of this SP pathway in response to various physiological and pharmacological treatments to determine the directionality and extent of neuronal feedback signals. Moreover, these cannulation and labeling proce-

dures may be of general interest and utility in anatomical studies examining neuronal circuits within the CNS as well as in biochemical studies examining protein and peptide processing reactions in discrete neuronal projections.

Note added in proof. Since the submission of this paper, Nawa et al. (1983) have published the nucleotide sequences of cloned recombinant cDNA's for two types of bovine striatal substance P precursor.

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