Active Uptake System for Substance P Carboxy-Terminal Heptapeptide (5-11) into a Fraction from Rabbit Enriched in Glial Cells

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Abstract—In the present study, we demonstrated the existence of an active uptake system for substance P carboxy-terminal heptapeptide, (5–11)SP. When a fraction from rabbit brain enriched in glial cells was incubated with [3H](5–11)SP, an uptake of [3H](5–11)SP was observed. The uptake system has the properties of an active transport mechanism. Kinetic analysis indicated two components of [3H](5–11)SP uptake, one representing a high and the other a low affinity transport system. After unilateral ablation of the striatum, approximately 30% of the high affinity [3H](5–11)SP uptake capacity of substantia nigra slices disappeared. The subcellular distribution of the high affinity uptake indicated that [3H]5-hydroxytryptamine was taken up mostly into the P2B fraction (synaptosomal fraction), whereas [3H](5–11)SP was taken up into the P2A fraction (myelin fraction) to the same extent as into the P2B fraction. These results suggest that when SP is released from nerve terminals, it is hydrolysed into (5–11)SP, which is in turn accumulated into glial cells as well as nerve terminals and that this high affinity uptake mechanism may play an important role in terminating the synaptic action of SP.

The presence of substance P (SP) in the central nervous system (CNS) has focused attention on a putative neurotransmitter or neuromodulator role of this peptide. We have previously reported that nerve terminals and glial cells lack an active uptake system for SP capable of terminating a possible transmitter action of SP (1, 2). However, when slices from either rat brain or rabbit spinal cord were incubated with labeled SP carboxy-terminal heptapeptide, [3H](5–11)SP, an uptake of [3H](5–11)SP into slices was observed (3). The uptake system has the properties of a high affinity, energy-dependent transport mechanism. Since a postproline cleaving enzyme that produces (5–11)SP was found in bovine (4) and rat brain (5) and the existence of (5–11)SP was demonstrated in the pig brain (6), we suggested that such an uptake system may have an important role in terminating a synaptic action of SP. However, the regional distribution of [3H](5–11)SP uptake in brain did neither correlate with that of SP nor with that of SP specific binding to synaptic membranes (Y. Nakata, Doctor Dissertation). This indicates that (5–11)SP uptake is not specifically localized in areas rich in SP-containing nerve terminals.

Several studies have demonstrated that glial cells also participate in the termination of the transmitter action of γ-aminobutyric acid (GABA) (7, 8). In the present work, we examined the ability of a fraction from rabbit brain enriched in glial cells to accumulate [3H](5–11)SP and demonstrated the existence of a high affinity, active uptake system similar to that observed in brain slices.
Materials and Methods

Purification of [3H](5-11)SP: (5-11)SP, synthesized by the conventional method described in a previous paper (9), was labeled with tritium by a catalyzed gas-exposure method, using platinum as the catalyst, and purified by the method described previously (3). The specific activity was 2.55 Ci/mmol.

Preparation of brain slices: Rabbit brain slices were prepared by the method described previously (3).

Preparation of fractions enriched in glial cells: Fractions enriched in glial cells were prepared according to the method of Nagata et al. (10) with a slight modification described in detail elsewhere (2).

Uptake of [3H](5-11)SP: The glial cell enriched fraction was suspended in 95% O2-5% CO2 saturated modified Krebs solution, which was composed of 126.5 mM NaCl, 2.4 mM KCl, 0.83 mM MgCl2, 1.1 mM CaCl2, 0.5 mM Na2SO4, 27.5 mM NaHCO3, 0.5 mM KH2PO4 and 5.9 mM glucose, which additionally contained 0.5 unit/ml aprotinin (Takeda Pharmaceutical Co., Japan) and 17 units/ml bacitracin (Sigma Chemical Co., St. Louis, MO) to inhibit the peptidase that might be present.

Experiments were carried out by the method described previously (1). Portions of tissue suspension containing about 1-1.5 mg protein were transferred into 10 ml polyethylene tubes. After preincubation for 10 min at 37°C or 0°C, 0.1 ml of [3H](5-11)SP (final concentration given in results) was added, and a further incubation was carried out. At the end of this incubation, 3 ml of cold saline was added to the reaction mixture which was then centrifuged at 15,000 g for 10 min at 4°C. The supernatant fluid was discarded. The pellet was resuspended in 4 ml of cold saline and recentrifuged at 15,000 g for 10 min. After centrifugation, the pellet was incubated in 0.1 ml 1M NaOH for 60-120 min at 37°C. After neutralization with 0.1 ml of 1M HCl, the radioactivity was determined in 10 ml Bray’s solution by a model 3320 Packard Tri-Carb liquid scintillation spectrometer and corrected for efficiency by external standardization. Counting efficiency was about 35%. The net uptake was calculated by subtracting the value at 4°C from the value at 37°C.

Identification of [3H](5-11)SP: Tritium activity in the pellet was extracted with 0.1 M HCl by boiling at 100°C for 10 min. The extract was centrifuged at 100,000 g for 10 min, after which the supernatant was lyophilized. Thereafter, the tritium activity was subjected to TLC on silica gel H (HPTLC Fertigplatten, Kieselgel 60, Merck GmH). After development, the chromatogram was divided into 10-12 zones. Each zone was scratched out and the radioactivity measured. Authentic (5-11)SP in 0.1 M acetic acid was also subjected to TLC, and the spot was located by spraying with 0.02% fluorescamine solution in acetone. The light blue fluorescence was visible when irradiated by ultraviolet light (360 nm).

In some experiments, the tritiated radioactivity from the tissue was analyzed by HPLC. The chromatography was carried out on an ODS-silica (TSK-GEL LS 410) column (300x4 mm internal diameter) with a model 6000A pump, a model U6K injector (Waters Associate, Milford, MA) and a model SPD-1 spectrophotometric detector (Shimadzu Seisakusho Ltd., Kyoto, Japan) with a dual-pen recorder. The mobile phase was methanol: water: trifluoroacetic acid (450:550:1, by vol.). The flow rate was 0.5 ml/min and the peptides were detected by UV absorbance at 210 nm, and 60-drop fractions were collected for liquid scintillation counting in Bray’s solution at 35% efficiency.

Unilateral ablation of the striatum: Rabbits weighing 2-3 kg were anesthetized by intravenous injection of 50 mg/kg of sodium pentobarbital (Nembutal, Abbott Lab.) and placed on a stereotaxic instrument. A hole was drilled in the skull at 3-9 mm lateral to the midline and 3-11 mm anterior to the bregma. After the deflection of the dura, the striatum of one side was aspirated by a cannula (1.2 mm in diameter) from a depth of 6 mm to a depth of 9 mm from the surface. In order to prevent post-operative adhesion, the lesion site was wrapped with spongel (Yamanouchi Pharmaceutical Co., Ltd.) and the flap was sutured. The extent of the lesion was verified histologically at the conclusion
of the experiments.

The substantia nigra was isolated 8–14 days after operation, divided into a right and left half and dissected with a razor blade into cubic slices. These slices were suspended in ice-cold modified Krebs-Ringer solution and allowed to stand for several minutes. After removal of the supernatant by decantation, the suspension was sieved through Nylon mesh with a pore size of 680 μm, and the resulting filtrate was used for the study.

**Subcellular distribution of [3H](5-11)SP uptake:** Rabbit brain stem and spinal cord were removed, homogenized in 10 vol. of 0.32 M sucrose in a Teflon-glass homogenizer and centrifuged at 1,000 g for 10 min. The supernatant was centrifuged at 17,000 g for 20 min, and the resulting pellet fraction (crude mitochondrial P2 fraction) was suspended in modified Krebs solution and incubated with [3H](5-11)SP as described above. At the end of the incubation periods, the reaction mixture was centrifuged at 17,000 g for 20 min, after which the pellet was resuspended in 0.32 M sucrose solution (approximately 2 ml for every gram of original tissue). The method of subsequent subfractionation was similar to that described by Gray and Whittaker (11). Five ml of this suspension was laid on the top of a discontinuous density gradient consisting of 12 ml each of 0.8 M and 1.2 M sucrose per tube and centrifuged at 53,500 g for 120 min. This resulted in the subfractions P2A, P2B and P2C which contained predominantly myelin, synaptosomes and mitochondria, respectively. Each layer was carefully separated, and the radioactivities and the protein amounts of each fraction were determined. As a reference, the P2 fraction was incubated with [3H]5-hydroxytryptamine ([3H]5-HT) and subfractionated as described above.

**Results**

**Identification of [3H](5-11)SP:** When the radioactivity extracted from the fraction enriched in glial cells incubated with [3H](5-11)SP was subjected to TLC and the chromatogram was developed to 10 cm from the origin, two peaks (Rf 0.78, 0.44) of radioactivity were observed (Fig. 1). The major peak, comprising approximately 50% (37°C) or 30% (4°C) of the total radioactivity recovered, was found at the zone corresponding to authentic (5-11)SP. Another minor peak was obtained at a zone 5 cm from the origin. The identity of the substance at this zone remains to be determined.

The radioactivity extracted by boiling with 0.1 M HCl from the fraction enriched in glial cells incubated with [3H](5-11)SP at 37°C was centrifuged at 100,000 g for 10 min, and the supernatant was filtered with Ultrafree (Worthington, Freehold, NJ) to remove the high molecular proteins. The filtrate was lyophilized and dissolved in HPLC buffer and injected into the HPLC system. The eluate at the zone corresponding to authentic (5-11)SP was pooled and reanalyzed by HPLC. The radioactivity, which corresponds to approximately 30% of the original radioactivity, gave a single peak that coincided with (5-11)SP (Fig. 2), confirming its identity with authentic (5-11)SP.
Fig. 2. HPLC of [3H] (5-11)SP. The radioactivity was extracted from fractions enriched in glial cells incubated with [3H] (5-11)SP at 37°C for 10 min and was filtered through Ultrafree, after which the filtrate was subjected to HPLC. Authentic SP and (5-11)SP were subjected to HPLC and the peptides were detected by UV absorbance at 210 nm (upper). For details, see Materials and Methods.

Fig. 3. Uptake of [3H] (5-11)SP into fractions enriched in glial cells as a function of the time of incubation. The fractions were incubated with 4.7x10⁻⁸ M [3H] (5-11)SP for 5-40 min at 37°C and 4°C and assayed for tritium content. As shown in Fig. 3, net uptake calculated by subtracting the value at 4°C from the value at 37°C was linear for about 10 min, after which the rate of uptake declined rapidly and reached a plateau by 20 min. Uptake of [3H] (5-11) SP was proportional to the protein concentration in the medium up to 1.7 mg/0.9 ml (Fig. 4). Fig. 5 shows the effect of pH on [3H] (5-11)SP uptake into the glial cell fraction. The fraction had a pH optimum at about 7.4.

To determine if [3H] (5-11)SP uptake into the glial cell fraction is an energy requiring process as it is the case in slices, the following experiments were performed. To 0.8 ml of the fraction suspension, 0.1 ml of ouabain (final concentration, 10⁻⁴ M), dinitrophenol (DNP) (final concentration, 10⁻³ M), synthetic (5-11)SP (final concentration, 12.5 nM) or H₂O for the control was added and incubation was carried out for 10 min at 37°C, after which 0.1 ml of [3H]
(5-11)SP was added and a further incubation was carried out for 10 min at 37°C. After a 10 min incubation period, [3H](5-11)SP uptake was reduced significantly by ouabain, DNP or (5-11)SP (Fig. 6). When the fractions were preincubated at 0°C or 25°C for 10 min and the incubation was continued with [3H](5-11)SP for a further 10 min at 0°C or 25°C, uptake of [3H](5-11)SP was significantly reduced when compared with that at 37°C (Fig. 6). When Na⁺ in the reaction mixture had been replaced with Tris-chloride or when the fractions were osmotically shocked by being dispersed in ice-cold H₂O, [3H](5-11)SP uptake was also reduced significantly.

**Fig. 4.** Effect of protein amount on [3H](5-11)SP uptake into fractions enriched in glial cells. The fractions containing 0.2-5 mg protein/0.9 ml were incubated with 4.7x10⁻⁸ M [3H](5-11)SP for 10 min at 37°C.

**Fig. 5.** Effect of pH on [3H](5-11)SP uptake into fractions enriched in glial cells. The fractions were incubated with 4.7x10⁻⁸ M [3H](5-11)SP. The different pH values were achieved by adding 0.1 M HCl to the incubation medium. Each value represents the mean ± S.E.M. of 3 experiments.

**Fig. 6.** Inhibition of [3H](5-11)SP uptake into fractions enriched in glial cells. Control and samples with drugs were preincubated at 37°C or at the indicated temperature for 10 min, after which 4.7x10⁻⁸ M [3H](5-11)SP was added, and a further incubation was carried out for 10 min at 37°C or at the indicated temperature (For details, see the text). Effect of removal of Na⁺ was examined by replacing NaCl in the incubation medium with Tris-chloride. Samples with hypoosmotic treatment were incubated with [3H](5-11)SP at 37°C for 10 min, after which the fractions were osmotically shocked by being dispersed in ice-cold H₂O.
reduced significantly (Fig. 6).

To evaluate the kinetics of \(^{[3]H}(5-11)\)SP uptake, the fractions were incubated with concentrations of \(^{[3]H}(5-11)\)SP varying from \(1.38 \times 10^{-8} \text{ M}\) to \(2.37 \times 10^{-7} \text{ M}\). \(^{[3]H}(5-11)\)SP uptake into the fractions enriched in glial cells was saturable and biphasic (Fig. 7). Analysis by Lineweaver-Burk plots showed two linear components. The \(K_m\) and \(V_{max}\) values for the high affinity component were \(3.25 \times 10^{-8} \text{ M}\) and 12.8 fmol/mg protein/min, respectively, and those for the low affinity component were \(4.52 \times 10^{-7} \text{ M}\) and 93.5 fmol/mg protein/min, respectively (Fig. 8).

Unilateral ablation of the striatum: The question as to whether the high affinity \(^{[3]H}(5-11)\)SP uptake system into slices is specifically localized in presumed SP-containing nerve terminals was studied by ablation experiments. When the striatum was removed unilaterally, in the ipsilateral substantia nigra slices, the \(K_m\) and \(V_{max}\) values for the high affinity component were \(8.77 \times 10^{-8} \text{ M}\) and 18.1 fmol/10 mg wet weight/min, respectively, and those in the contralateral side were \(1.25 \times 10^{-7} \text{ M}\) and 27.0 fmol/10 mg wet weight/min, respectively (Fig. 9). The difference between these two \(V_{max}\) values was statistically nonsignificant.

Subcellular distribution of \(^{[3]H}(5-11)\)SP uptake: Figure 10 shows the subcellular distribution of \(^{[3]H}\)ligand uptake. \(^{[3]H}\)5-HT uptake was predominantly located in the synaptosomal P2B fraction from rabbit brain stem or spinal cord and to some extent in the myelin P2A fraction, while \(^{[3]H}(5-11)\)SP was accumulated into the myelin P2A fraction to the same extent as into the synaptosomal P2B fraction from rabbit brain stem.

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**Fig. 7.** Saturation curve of \(^{[3]H}(5-11)\)SP uptake into fractions enriched in glial cells. The fractions were incubated with \(1.38 \times 10^{-8} \text{ M} - 2.37 \times 10^{-7} \text{ M}\) \(^{[3]H}(5-11)\)SP at 37°C for 10 min. Each point represents the mean value of 2–5 experiments.

**Fig. 8.** Lineweaver-Burk plot for \(^{[3]H}(5-11)\)SP uptake into fractions enriched in glial cells. The fractions were incubated with \(1.38 \times 10^{-8} \text{ M} - 2.37 \times 10^{-7} \text{ M}\) \(^{[3]H}(5-11)\)SP at 37°C for 10 min. Each point represents the mean value of 2–5 experiments.
Discussion

We have previously reported that $[^3\text{H}](5-11)$SP was accumulated by slices from either rat brain or rabbit spinal cord by a high affinity, energy-dependent transport mechanism (3). However, in these complex tissues, the respective involvement of neuronal and glial cells in the uptake could not be separated. The regional distribution of $[^3\text{H}](5-11)$SP uptake correlates neither with that of SP content nor with that of SP specific binding to synaptic membranes (Y. Nakata, Doctor Dissertation). These results indicate that $[^3\text{H}](5-11)$SP uptake is not specifically localized in presumed SP-containing nerve terminals. It has become increasingly evident that glial cells may participate in the termination of the transmitter action of GABA (7, 8). Therefore, we examined the ability of glial cells to accumulate $[^3\text{H}](5-11)$SP.

In the present study, fractions enriched in glial cells were prepared by the method of Nagata et al. (10). The fractions are contaminated with a few neurons (10). However, we previously observed that GABA, which is known to be accumulated mostly into glial cells, was taken up actively into glial fractions, and in contrast, 5-HT, which is known not to be accumulated into glial cells, was not taken up into the fractions (2). Therefore, with respect to the glial uptake experiment, the contamination of glial fractions with neuronal components is thought not to be significant. Using these fractions we demonstrated the existence of an active uptake system for $[^3\text{H}](5-11)$SP similar to that observed in brain slices. It was
temperature and Na\(^+\) dependent and inhibited by the addition of ouabain, DNP and synthetic (5-11)SP. Therefore, it appears that \([^{3}\text{H}] (5-11)\text{SP}\) is transported into glial cells by an energy-dependent mechanism. Double reciprocal plots for the \([^{3}\text{H}](5-11)\text{SP}\) uptake into glial cells yield two linear components. The \(K_m\) value of the high affinity uptake component in glial cells was nearly the same as that previously reported for \([^{3}\text{H}] (5-11)\text{SP}\) uptake into brain slices (3).

After striatal ablation, a slight reduction of the high affinity \([^{3}\text{H}](5-11)\text{SP}\) uptake capacity of substantia nigra slices was observed. Since the striato-nigral fiber system has been found to contain SP fibers (12), the lesion-sensitive \([^{3}\text{H}](5-11)\text{SP}\) uptake sites in the substantia nigra might be localized in SP-containing nerve terminals, while the lesion-resistant uptake might represent glial \([^{3}\text{H}](5-11)\text{SP}\) uptake as well as uptake into neurons other than SP-containing fibers.

Finally, the subcellular distribution of \([^{3}\text{H}](5-11)\text{SP}\) uptake was examined. In contrast to \([^{3}\text{H}]5\text{-HT}\) which was taken up mostly into the synaptosomal fraction, \([^{3}\text{H}](5-11)\text{SP}\) was taken up into the myelin fraction derived from oligodendroglia, to the same extent as into the synaptosomal fraction. Because regional distribution of \([^{3}\text{H}](5-11)\text{SP}\) uptake into rabbit brain and spinal cord slices correlated with neither that of SP level nor that of SP specific binding to the synaptic membranes (Y. Nakata, Doctor Dissertation) and in addition, striatal ablation could not significantly affect the high affinity \([^{3}\text{H}](5-11)\text{SP}\) uptake in substantia nigra, part of the synaptosomal uptake in subcellular distribution might reflect \([^{3}\text{H}](5-11)\text{SP}\) uptake into nerve terminals other than SP-containing neurons.

At present, the physiological significance of this high affinity uptake mechanism into glial cells is far from clear. However, recently the existence of a postproline cleaving enzyme capable of degrading SP into (5-11) SP and the presence of (5-11) SP in CNS have been reported (4-6). Therefore, the possibility can not be ruled out that such a high affinity uptake mechanism may play an important role in terminating the synaptic action of SP. The nature of the low affinity uptake remains to be determined.

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References
1 Segawa, T., Nakata, Y., Yajima, H. and Kitagawa, K.: Substance P in the central nervous system of rabbit: Uptake system differs from putative transmitter. Japan. J. Pharmacol. 26, 757–760 (1976)
2 Segawa, T., Nakata, Y., Yajima, H. and Kitagawa, K.: Further observation on the lack of active uptake system for substance P in the central nervous system. Japan. J. Pharmacol. 27, 573–580 (1978)
3 Nakata, Y., Yajima, H. and Segawa, T.: Active uptake of substance P carboxy-terminal heptapeptide (5-11) into rat brain and rabbit spinal cord slices. J. Neurochem. 37, 1529–1543 (1981)
4 Blumberg, S., Teichberg, V.I., Charli, J.L., Hersh, L.B. and McKelly, J.F.: Cleavage of substance P to an N-terminal tetrapeptide and a C-terminal heptapeptide by a post-proline cleaving enzyme from bovine brain. Brain Res. 192, 477–486 (1980)
5 Kato, T., Nakano, T., Kojima, K., Nagatsu, T. and Sakakibara, S.: Change in prolyl endopeptidase during maturation of rat brain and hydrolysis of substance P by the purified enzyme. J. Neurochem. 35, 527–535 (1980)
6 Kato, T., Okada, M., Nakano, T., Nagatsu, T., Emura, J., Sakakibara, S., Iizuka, Y., Tsushima, S., Nakazawa, N. and Ogawa, H.: The presence of substance P carboxy-terminal heptapeptide in pig brain stem. Proc. Japan. Acad. 56, 388–393 (1980)
7 Henn, F.A. and Hamberger, A.: Glial cell function: Uptake of transmitter substances. Proc. Nat. Acad. Sci. 68, 2686–2690 (1971)
8 Schon, F. and Kelly, J.S.: The characterization of \([^{3}\text{H}]\text{GABA}\) uptake into the satellite glial cells of rat sensory ganglia. Brain Res. 66, 280–300 (1974)
9 Kitagawa, K., Ujita, K., Kiso, Y., Akita, T., Nakata, Y., Nakamoto, N., Segawa, T. and Yajima, H.: Synthesis and activity of C-terminal heptapeptides of tachykinins and bombesin-like peptides. Chem. Pharm. Bull. (Tokyo) 27, 48–57 (1979)
10 Nagata, Y., Mikoshiba, K. and Tsukada, Y.: Neuronal cell body enriched and glial cell enriched fractions from young and adult rat
brain: Preparation and morphological and biochemical properties. J. Neurochem. 22, 493–503 (1974)

11 Gray, E.G. and Whittaker, V.P.: The isolation of nerve endings from brain: An electron-microscopic study of cell fragments derived by homogenization and centrifugation. J. Anat. 96, 79–88 (1962)

12 Kanazawa, I., Emson, P.C. and Cuello, A.C.: Evidence for the existence of substance P-containing fibers in striato-nigral and pallido-nigral pathways in rat brain. Brain Res. 119, 447–453 (1977)