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Direct projections from substance P-containing neurons to nitric oxide synthase-containing interneurons in the rat striatum

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Abstract

We reported previously that substance P (SP)-containing projection neurons (SP-PN) in the striatum emitted many axon collaterals within the striatum (J. Comp. Neurol. 388 (1997) 250), and that substantially all striatal interneurons showing immunoreactivity for nitric oxide synthase (NOS: synthetic enzyme for the freely-diffusible messenger nitric oxide) displayed immunoreactivity for SP receptor (NK1: NK-1-type tachykinin receptor; Neurosci. Lett. 310 (2001) 109). By combining immunohistochemistry for NOS with immunogold labeling for SP, the present study revealed that SP-immunoreactive axon terminals were in synaptic contact with NOS-immunoreactive aspiny neurons in the rat striatum, indicating that SP-PN in the striatum sent their axon collaterals to nitric oxide synthase-expressing interneurons (NOS-IN) in the striatum. On the basis of these present and previous data, possible synaptic and non-synaptic interactions between SP-PN and NOS-IN in the striatum were discussed.

Keywords: Striatum; Nitric oxide synthase; Substance P; Neurokinin-1 receptor; Nitric oxide; Immunohistochemistry; Rat

The free radical gas nitric oxide (NO) is a freely-diffusible neuronal messenger that performs diverse signaling functions in the nervous system [7,11,18]. The synthetic enzyme for NO (nitric oxide synthase (NOS)) in neurons is a Ca^{2+}/calmodulin-dependent enzyme. When the local Ca^{2+} concentration is elevated, NOS converts arginine to citrulline, generating NO. The NO production by NOS in neurons occurs almost exclusively following activation of *N*-methyl-D-aspartate-type glutamate receptors, to which NOS is connected via postsynaptic density protein (PSD-95) [3,8].

In the striatum, NOS is expressed in one class of interneurons (for review, see [10]). These interneurons receive glutamatergic, GABAergic and dopaminergic projection fibers, respectively, from the cerebral cortex, globus pallidus and substantia nigra pars compacta, and send their axons to GABAergic striatal projection neurons which contain substance P (SP) or enkephalin (for review, see [10]); the vast majority of them express somatostatin and/or neuropeptide Y [6,17].

We reported previously that substance P-containing projection neurons (SP-PN) in the striatum emitted many axon collaterals within the striatum, and that almost all nitric oxide synthase-expressing interneurons (NOS-IN) in the striatum displayed receptor for SP (NK1: NK-1-type tachykinin receptor) [14]. This indicated that SP-PN in the striatum sent SP-signals to NOS-IN via their intrastriatal axon collaterals. However, SP might act as a paracrine neuromediator in the striatum; SP-signals in the striatum might be mediated not only synaptically but also non-synaptically [12,13]. Thus, in the present study, we examined, using light and electron microscopy, if NOS-IN in the striatum might be in synaptic contact with SP-containing axon terminals.

Six adult male Wistar rats weighing 200–250 g were used. All procedures for the experiments were approved by the Animal Care and Use Committees at the Fourth Military Medical University and at the Graduate School of Medicine, Kyoto University. The rats were anesthetized by intraperitoneal injection of an overdose of sodium pentobarbital (100 mg/kg body weight). The anesthetized rats were perfused transcardially with 150 ml of 25 mM phosphate-buffered 0.9% saline (PBS; pH 7.3), followed with 500 ml of 0.1 M phosphate buffer (PB; pH 7.3) containing 4% (w/v) paraformaldehyde and 75% (v/v)-saturated picric acid. After the

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perfusion, the brain was removed immediately and placed in 0.1 M PB containing 25% (w/v) sucrose overnight at 4 °C. Then the brains were cut serially into 25 μm thick frontal sections on a freezing microtome. The sections through the striatum were collected consecutively in two series. One series of the sections was processed for double-immunofluorescence histochemistry for NOS and SP. Briefly, the sections were incubated at room temperature sequentially with: (1), a mixture of 0.75 μg/ml anti-SP rabbit serum (1:1500; Incstar, Stillwater, MN) and 1 μg/ml anti-NOS sheep IgG (1:1000; Chemicon, Temecula, CA) overnight; (2), 10 μg/ml biotinylated anti-sheep IgG donkey antibody (Chemicon) for 3 h; (3), a mixture of 10% (v/v) normal sheep serum, 10 μg/ml dichlorotriazinylaminofluorescein (DTAF)-labeled anti-rabbit IgG donkey antibody (Chemicon), and 5 μg/ml Texas Red-labeled avidin D (Vector, Burlingame, CA) for 4 h. The incubation medium was prepared by using 0.05 M PBS containing 0.5% (v/v) Triton X-100, 0.25% (w/v) L-carrageenan, 0.05% (w/v) NaN_3, and 5% (v/v) normal donkey serum in step (1) and step (2), and by using 0.05 M PBS containing 0.3% (v/v) Triton X-100 in step (3).

After incubation, the sections were rinsed in 0.05 M PBS, mounted onto gelatin-coated glass slides, air-dried, coverslipped with a mixture of 50% (v/v) glycerin and 2.5% (w/v) triethylene diamine (anti-fading reagent) in 0.05 M PBS, and observed with an epifluorescence microscope (BX-60; Olympus, Tokyo, Japan) under the appropriate filter for Texas Red (excitation, 530–585 nm; emission, ≥610 nm) and for DTAF (excitation, 450–490 nm; emission, 515–565 nm). The sections were further observed with a confocal laser-scanning microscope (Bio-Rad MRC-1024, Hercules, CA) by using laser beams of 568 and 488 nm with the appropriate emission filter for Texas Red (570–610 nm) and DTAF (510–550 nm).

Another series of the sections were used for the control experiments for the double-immunofluorescence histochemistry for NOS and SP. When the primary antibody for NOS or that for SP was omitted or replaced with normal IgG, no immunoreactivity for the omitted or replaced antibody was detected.

For electron microscopic study, the anesthetized rats were perfused transcardially with 100 ml of 0.025 M PBS (pH 7.4), followed with 500 ml of 0.1 M PB (pH 7.4) containing 4% (w/v) paraformaldehyde, 0.01% glutaraldehyde and 15% (v/v)-saturated picric acid. After the perfusion, the brain was removed immediately, placed in 0.1 M PB (pH 7.4), and cut into 50 μm thick frontal sections on a Vibratome (Microslicer; Dosaka, Kyoto, Japan). The sections through the striatum were placed in 0.05 M PB containing 25% sucrose and 10% (v/v) glycerol for 30 min for cryoprotection. Then, the sections were freeze-thawed with liquid nitrogen for enhancement of penetration of antibodies in the following immunocytochemical reaction. Then the sections were placed in 50 mM Tris-buffered saline (TBS; pH 7.4) containing 20% normal goat serum for 1 h to block non-specific immunoreactivity.

For electron microscopic double-immunocytochemistry, the immunogold–silver method for SP was combined with the immunoperoxidase method for NOS. The sections were incubated with a mixture of anti-SP rabbit serum (1:1000; Incstar) and anti-NOS sheep IgG (1:500; Chemicon) for 24 h at room temperature; the incubation medium was 0.05 M TBS containing 2% (v/v) normal goat serum (TBS-NGS). Then the sections were washed in TBS and incubated at room temperature for 14–16 h with a mixture of biotinylated anti-[sheep IgG] donkey antibody (Chemicon) and anti-[rabbit IgG] goat antibody conjugated to 1.4 nm gold particles (Nanoprobes; Stony Brook, NY); each was diluted at 1:100 in the TBS-NGS. Subsequently the sections were treated as described elsewhere [13]. Briefly, the sections were processed for: (1), postfixation with glutaraldehyde; (2), silver enhancement with HQ Silver Kit (Nanoprobes); (3), incubation with ABC Kit (Vector, Burlingame, CA); (4), visualization of NOS-immunoreactivity with diaminobenzidine tetrahydrochloride and H_2O_2; (5), osmification; (6), counterstaining with uranyl acetate; and (7), flat-embedding in Durcupan (Fluka, Buchs, Switzerland) after dehydration and mounting on silicon-coated slide glass. Subsequently, the parts of the striatum containing many silver-intensified SP-positive axons and NOS-immunoreactive cells were trimmed from the flat-embedded tissue specimen under an operation microscope and cut into ultrathin sections. The ultrathin sections were mounted on single-slot grids coated with pioloform membrane and examined with an electron microscope (CM 100; Philips, Eindhoven, Netherlands).

Under a confocal laser-scanning microscope, NOS-positive neurons and SP-positive axonal components were identified with red fluorescence of Texas Red and with green fluorescence of DTAF, respectively (Fig. 1). Many SP-positive axonal varicosities were scattered throughout the striatum. NOS-positive neurons were aspiny neurons with the maximal soma diameters ranging from 10 to 25 μm. SP-positive axonal varicosities in close apposition to NOS-positive neurons were seen occasionally on cell bodies (Fig. 1a) and frequently on dendrites (Fig. 1b), although the distribution density of SP-positive axonal varicosities on each NOS-positive neuronal profiles was not so high.

Electron microscopically, NOS-positive neuronal profiles contained reaction products of the immunohistochemistry (electron-dense, flocculent precipitates), while SP-positive axonal profiles were labeled with silver grains (Fig. 2). SP-positive axon terminals usually contained pleomorphic synaptic vesicles. Most SP-positive synaptic terminals on NOS-positive neurons were seen on dendritic profiles. SP-positive axons appeared to make synapses of the symmetric type on NOS-positive neurons, although immunoperoxidase reaction products in postsynaptic areas often made it difficult to judge properly the type of synapse.

The results of the present study indicated that NOS-IN in the striatum were in synaptic contact with SP-containing axon terminals, which were most likely those of collaterals of SP-PN in the striatum [12]. SP might exert influences

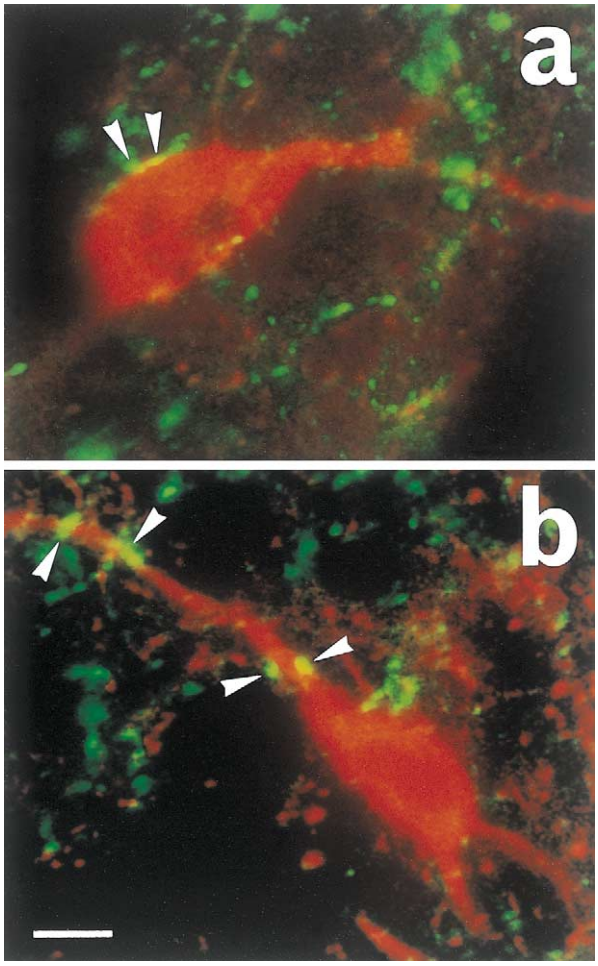

Fig. 1. Digital images of two sections through the striatum of a rat, taken with a confocal laser-scanning microscope. NOS-immunoreactivity is visualized with red fluorescence of Texas Red, while SP-immunoreactivity is visualized with green fluorescence of DTAF. Arrowheads indicate SP-positive axonal components in close apposition to a cell body (a) or a dendrite (b) of NOS-positive neurons. Scale bar, 3.5 μm.

upon NK1 on NOS-IN in the striatum [12,13]. NK3 (NK3-type tachykinin receptor) was also indicated to be expressed in striatal NOS-IN; SP might have weak effect at NK3 on NOS-IN in the striatum [16]. On the other hand, NOS-IN in the striatum have been indicated to send their axons to striatal projection neurons [4,15]. Thus, reciprocal synaptic connections are considered to exist between NOS-IN and SP-PN in the striatum.

However, NO is a freely-diffusible messenger, which may diffuse from the site of production in the absence of any specialized release machinery [7,18]. Even without axonal projections from NOS-IN to SP-PN in the striatum, NO may cross cell membranes of SP-PN, since striatal projection neurons are enriched with guanylate cyclase [2] (guanylate cyclase is a second messenger associated with NO signaling; for review, see [5,19]). In fact, newly synthesized NO in the NO-producing cells in the spinal dorsal horn has been indicated to trigger the release of SP from primary

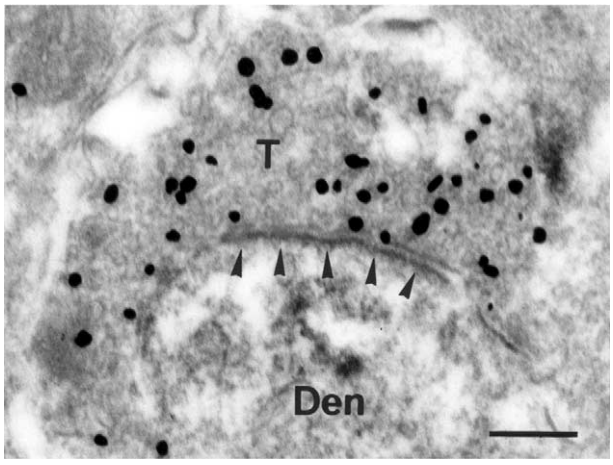

Fig. 2. An SP-positive axon terminal (T) labeled with silver grains is in synaptic contact with a NOS-positive dendritic profile (Den) containing electron-dense immunoreaction products. Arrowheads indicate synaptic site. Scale bar, 0.2 μm.

afferent axon terminals which are often in close spatial relationship with the NOS-producing cells [1] (also see [9]). SP has been reported to produce a prolonged elevation in Ca^{2+} concentration in the dorsal horn neurons by mobilizing calcium release from intracellular stores [20], and thus may possibly activate NOS.

In summary, the present results suggest that NOS-IN in the striatum are in synaptic contact with SP-containing axon terminals arising probably from striatal SP-PN. NO released from the NOS-IN in the striatum may exert influences upon striatal SP-PN.

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