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Short Communication

Immunodetection of heparin-binding growth associated molecule (pleiotrophin) in striatal interneurons

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Abstract

Pleiotrophin (PTN), a developmentally-regulated trophic factor, is over-expressed in the striatum of parkinsonian rats. Because striatal PTN can provide trophic support to dopamine neurons, we identified the cellular types containing PTN in the striatum of adult rats. By means of fluorescent double-immunolabeling, we found PTN to co-localize with a neuronal nuclei marker but not with glial fibrillary acidic protein. The number, distribution, and morphology of the PTN-immunolabeled cells suggested that they were interneurons. Further double-immunolabeling studies ruled out PTN localization to calretinin- and parvalbumin-containing interneurons. Instead, ~40% of the PTN-immunolabeled neurons contained nitric oxide synthase or somatostatin and ~60% expressed the vesicular acetylcholine transporter, supporting that they were GABAergic nitric oxide synthase/somatostatin-containing and cholinergic interneurons. Further work is necessary to determine if PTN from striatal interneurons can provide trophic support to dopamine neurons.

Theme: Development and regeneration
Topic: Neurotrophic factors: expression and regulation

Keywords: Pleiotrophin; Striatal interneuron; Immunohistochemistry; Parkinson's disease

Pleiotrophin (PTN) is a neurite outgrowth-promoting factor [20] and a mitogen isolated from bovine uterus [15] which is highly expressed in the nervous system and non-neural tissues during embryonic and early postnatal development [23]. PTN expression in the adult central nervous system is very low, but it can be induced in an activity-dependent manner [24] and after ischemic insults [22].

Recent studies revealed that PTN mRNA is increased in the striatum after nigrostriatal lesions [9] and chronic levodopa therapy [8]. As PTN promotes differentiation [9,16] of dopamine neurons in vitro, and increases the differentiation of embryonic stem cells to dopamine neurons [10], PTN might play an important role in nigrostriatal system development and in the compensatory mechanisms that take place in Parkinson's disease [4,17,25]. Therefore, it is important to determine what cellular elements express PTN in the basal ganglia. We have previously shown that unidentified cellular elements express PTN mRNA in the striatum [8]. Here, we performed Western blots and

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double-immunofluorescence on tissue sections to characterize PTN protein localization in the rat striatum.

Experiments were carried out on tissue samples from female Wistar rats (220–250 g) in compliance with the European Directive No.: 86/609/EEC. To obtain protein extracts for Western blot, the rats were anesthetized with ketamine and xylazine (40 mg/kg and 2 mg/kg i.p., respectively) and decapitated. The brain was removed and placed on a cold surface where the cerebral cortex and striatum were dissected out. The tissues were then placed in Tris 50 mM pH 7.4/NaCl 2 M containing a cocktail of protease inhibitors (Aprotinin 1 μg/ml, Leupeptins 1 μg/ml, Pepstatin A 1 μg/ml, PMSF 1 mM, EDTA 1 mM) and homogenized. Total protein concentration of the samples was measured using the BCA Protein Assay Kit (Pierce Biotechnology, USA). Western blots were performed on crude protein extracts (50 μg of total protein; Fig. 1a) and on a fraction of these extracts enriched in heparin-binding molecules (Fig. 1b). To concentrate the heparin-binding molecules, 200 μg of total protein (in 1 ml of Tris 50 mM pH 7.4/NaCl 0.5 M) was incubated at 4 °C overnight with 80 μl of heparin-conjugated Sepharose beads (10%, Amersham Biosciences, France), washed 3 times with 500 μl of Tris 50 mM pH 7.4/NaCl 0.5 M, then with Tris 50 mM pH 7.4. This procedure provided a very strong evidence that the antiserum has no cross-reactivity with other closely-related heparin-binding growth factors. The eluted proteins were electrophoresed on a 15% polyacrylamide gel and electrotransferred to an Immobilon-P membrane [16]. The membrane was incubated with the anti-PTN antiserum (rabbit polyclonal antibody directed against the amino acids 94–168 mapping at the carboxyl terminus of recombinant human PTN (rhPTN), 1:100, Santa Cruz Biotechnology, USA), a horseradish peroxidase-conjugated anti-immunoglobulin, and developed with standard chemiluminescent procedures. In order to test antiserum specificity, we exposed 3 ml of it (dilution 1:100) for 2 days to an Immobilon-P membrane containing 320 μg of rhPTN. Then, we eluted the specific anti-PTN antibodies which were used to demonstrate the presence of PTN in protein extracts (Fig. 1b).

The antiserum revealed, both in cortical and striatal samples, an 18 kDa molecular weight band that matched the molecular weight of rhPTN, and additional bands of about 36, 15, and 12 kDa, which were not observed in the lane loaded with rhPTN. Preadsorption of the anti-PTN antiserum abolished all staining, while incubation of the blotted membranes with purified anti-PTN antibodies revealed the presence of the 18 kDa band and to some extent the other bands only in the brain protein samples (Fig. 1b). These results suggest that the 36 kDa band is a PTN dimer and the low molecular weight bands represent truncated forms of PTN produced in vivo [2,6,14].

To obtain tissue for immunohistochemistry, 7 rats were anesthetized and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). The brain was postfixed for 2 h, cryoprotected in solutions with increasing sucrose concentration, and serially cut in 40-μm-thick coronal sections. The free-floating sections were incubated for 48 h at 4°C with the anti-PTN antiserum (1:100), rinsed in PBS, and incubated for 2 h at room temperature with a biotin-labeled anti-rabbit IgG antiserum (1:250; Vector Laboratories, USA). The primary antibody was visualized by means of an avidin–biotin peroxidase complex and 3,3′-diaminobenzidine [17]. Two control conditions were tested to determine the specificity of PTN labeling in brain sections. First, the primary antiserum was omitted from the experimental protocol leading to no tissue labeling (not shown). Second, the primary antiserum was exposed to membranes containing rhPTN (see above), then the antiserum, devoid of the anti-PTN antibodies, was used to label brain sections. Overall, PTN was present in 145 ± 29 cells (mean ± S.D, n = 7) per section per hemisphere (coronal plane 0.24 mm anterior from bregma, [19]) which were distributed throughout the striatum and had different neuron-like morphologies (Figs. 2a, b, c, d). Preadsorption of anti-PTN antibodies almost abolished the staining (Fig. 2f). Consistent with previous findings [5,21,23,24], PTN-immunoreactive neurons were found in

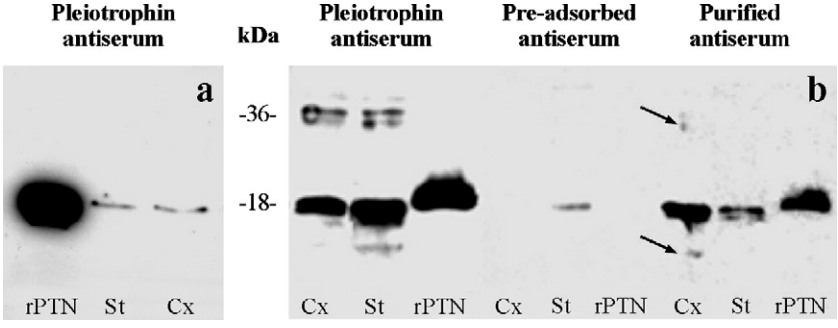

Fig. 1. Pattern of labeling produced by the anti-PTN antiserum in Western blot. Lanes contained crude proteins (a), extracted from cerebral cortex (Cx), striatum (St), or recombinant human pleiotrophin (rPTN, 50 ng). The antiserum weakly labeled an 18 kDa band corresponding to the PTN molecular weight. A similar blot, performed on extracts enriched in heparin-binding molecules (b), revealed some additional bands (left lanes). The pattern of labeling was almost abolished when the membrane was developed with an antiserum that was neutralized by previous incubation with rPTN (central lanes), and was reproduced by incubation with purified anti-PTN antibodies (right lanes). The arrows indicate weakly labeled bands.

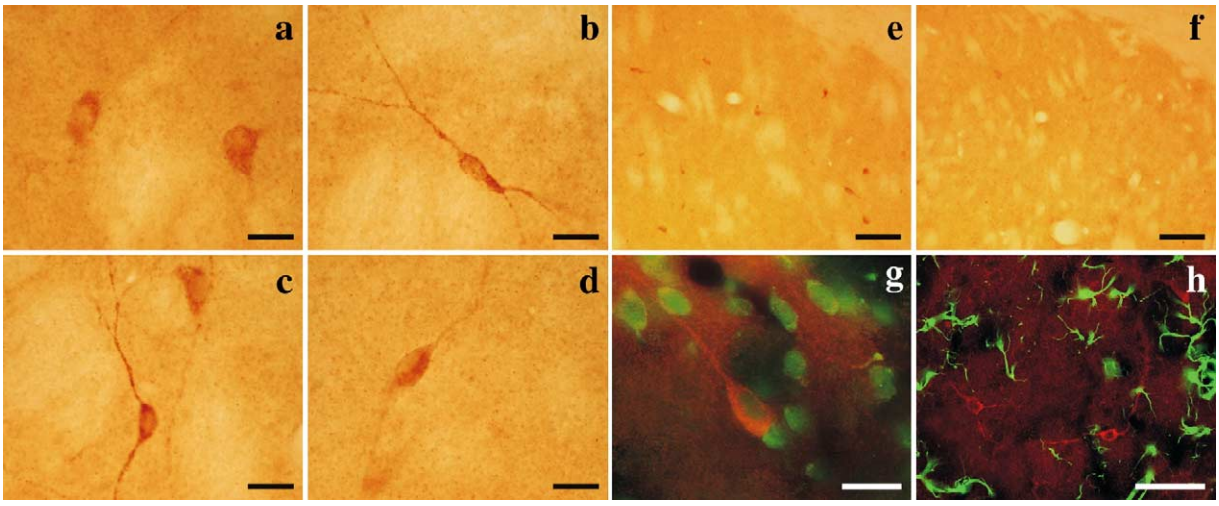

Fig. 2. The morphology and low density of striatal neurons labeled with the anti-PTN antiserum (a–d) suggested that they were interneurons. Some of them had a large rounded or polygonal soma from which few thick non-spiny dendrites emerged (a, d). The other PTN-immunoreactive neurons had smaller somata from which two or more non-spiny and long processes could be followed for a hundred micrometers or more from the soma emerged (b, c). To verify the specificity of tissue labeling, adjacent striatal sections were incubated with the anti-PTN antiserum (e) or with an aliquot of antiserum that was previously exposed to membranes blotted with rhPTN with the purpose of removing the specific anti-PTN antibodies from it (f). The procedure resulted in an almost complete disappearance of labeling. Double immunofluorescence for PTN (red) and the NeuN (green), or GFAP (green), in striatal sections showed that PTN was co-localized with NeuN (g) but not with GFAP (h). Scale bar: 20 μm (a–d); 100 μm (e, f); 20 μm (g); 50 μm (h).

the cerebral cortex and hippocampus and in glia-like cells in white matter tracts (not shown).

The morphology and distribution of labeled elements suggested that they were striatal interneurons. The main classes of striatal interneurons are the large cholinergic interneurons and GABAergic interneurons, the latter can in turn be sub-classified into parvalbumin-containing, nitric oxide synthase (NOS)/somatostatin (SST)/neuropeptide Y-containing, and calretinin-containing interneurons ([11]). To establish that the labeled cells were interneurons and further determine which ones were PTN-immunoreactive, we performed double-fluorescent immunolabeling for PTN plus an interneuronal marker in striatal sections adjacent to those stained with 3,3′-diaminobenzidine. We first ran the incubation with the anti-PTN antiserum, rinsed in PBS, and then incubated for 48 h at 4 °C with the second primary antiserum. PTN was visualized with biotin-labeled anti-rabbit IgG antiserum (Vector) and streptavidin-Cy3 (Sigma, USA), the other antigens with Alexa® 488 labeled antibodies (Molecular Probes, USA). The second primary antiserums were: (i) mouse anti-neuronal nuclei marker (NeuN, Chemicon, USA, 1:100); (ii) mouse anti-glial fibrillary acidic protein (GFAP, Sigma, 1:4000); (iii) goat anti-vesicular acetylcholine transporter (VAChT, Chemicon, 1:500); (iv) goat anti-SST (Santa Cruz, 1:500); (v) mouse anti-neuronal NOS (BD Transduction Laboratories, USA, 1:500); (vi) mouse anti-parvalbumin (Swant, Switzerland, 1:1000); and (vii) goat anti-calretinin (Chemicon, 1:500). As expected, all the PTN-immunoreactive cells contained NeuN (Fig. 2g) but not GFAP (Fig. 2h). Double immunolabeling (Fig. 3) further confirmed that they were interneurons. The PTN-immunoreactive neurons contained SST (range 33–45%), NOS (range 37–53%), or VAChT (range 60–63%) (Fig. 4a), but not calretinin or parvalbumin. Conversely, most VAChT-immunoreactive neurons (range 72–76%) and a minority of either SST- (range 15–20%) or

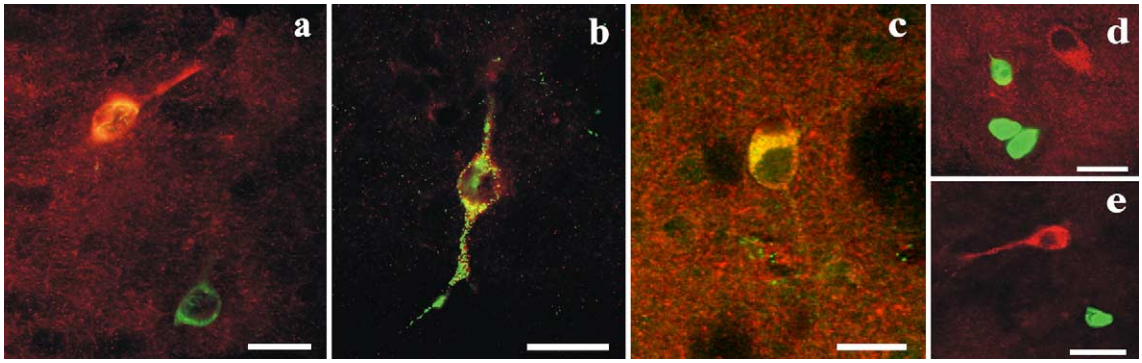

Fig. 3. Double immunofluorescence for PTN (red) and the interneuron markers (green) in striatal sections. PTN was co-localized with NOS (a), SST (b), and VAChT (c) but not with parvalbumin (d) and calretinin (e). Double-labeled neurons are yellow. Scale bar: 20 μm.

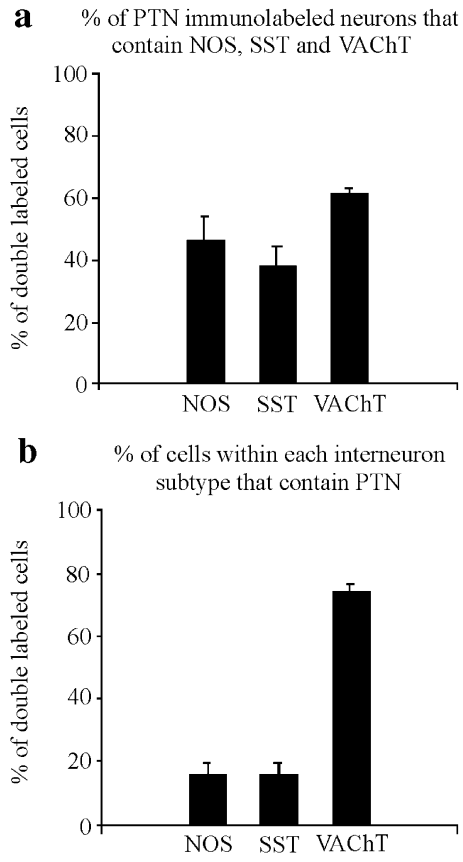

Fig. 4. Bar graphs showing the distribution of interneuron markers by PTN-immunoreactive striatal neurons (a) and of PTN within each striatal interneuron subpopulations (b). The proportion of labeled cells was estimated from direct counts performed on 10 non-overlaying 250× microscopic fields distributed along a single striatal section per rat (0.20 mm anterior from bregma, [19]; Mean ± SD; n = 3 rats).

NOS-positive neurons (range 12–18%) showed PTN immunoreactivity (Fig. 4b).

Since the discovery of PTN in 1989 [15,20], a body of work was devoted to describe its distribution in the embryo and neonate and to disclose its function during development, in the central nervous system as well as in many other tissues. However, very little is known about the localization and function of PTN in the adult brain. To our knowledge, this is the first report concerning the cellular localization of PTN in the rat striatum. The main finding was that PTN is present in two major classes of striatal interneurons, the cholinergic, and SST/NOS-containing GABAergic interneurons.

Two main neuronal types are present in the rat striatum, the output neurons with densely spiny dendrites, and the aspiny interneurons which represent less than 10% of all striatal neurons [11]. Striatal interneuron functions are poorly understood. The cholinergic interneurons are thought to correspond to the "tonically-active neurons" that have been implicated in learning mechanisms in the basal ganglia [1], while the GABAergic interneurons mediate feed-forward inhibition from the cortex to large networks of spiny projection neurons [12]. Very little is known about the effects of other putative interneuron neurotransmitters like SST, nitric oxide, and neuropeptide Y. Nitric oxide, acetylcholine, and GABA interact with dopamine to regulate long-term synaptic plasticity of corticostriatal connections [7]. Interestingly, PTN was reported to inhibit long-term potentiation in the hippocampus [18]. Given that PTN receptors are expressed in the adult striatum [9], it would be important to determine if striatal interneurons, through the release of PTN, can further modulate cortico-striatal long-term plasticity.

Two recent studies found increased expression of PTN mRNA in the striatum of 6-hydroxydopamine-lesioned rats (a widely used experimental model of parkinsonism) [8,9], where spontaneous and levodopa-induced partial recovery of dopamine innervation takes place [4,17]. Furthermore, PTN induces elongation and differentiation of dopamine neurites [9,16]. Together with the fact that cholinergic and GABAergic striatal interneurons express other neurotrophic factors, notably glial-derived neurotrophic factor [3], which has a powerful effect on dopamine neurons [13], it is tempting to speculate that striatal interneurons can stimulate the remodeling of dopamine innervation in physiological and pathological conditions.

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