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Differential gene expression induced by chronic levodopa treatment in the striatum of rats with lesions of the nigrostriatal system

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Abstract

Levodopa, the major treatment for patients with Parkinson's disease, has been shown to induce a variety of compensatory effects, including facilitation of sprouting by dopaminergic neurons, in experimental animals with lesions leading to denervation of the striatum. To better understand the cellular and molecular environment where most of these compensatory changes take place, in particular elements that might contribute to the recovery of dopaminergic innervation, we have constructed a differential expression library enriched in transcripts from the striata of rats with lesions of the medial forebrain bundle treated with levodopa for 6 months. We have used this library to screen an expression array of rat genes representing the major cell functions, and have identified several that are involved in neurotrophic mechanisms and plasticity. We have confirmed the differential expression of selected transcripts by non-radioactive *in situ* hybridization, and report that the growth factor pleiotrophin, myelin basic protein and calmodulin are overexpressed in the denervated striatum of levodopa-treated rats.

Keywords: calmodulin, macroarray, myelin basic protein, Parkinson's disease, plasticity, pleiotrophin.

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Levodopa is still the most effective pharmacological treatment for Parkinson's disease (PD). It produces a short-lived clinical response, the time-course of which is determined by plasma concentrations of the drug, and a long-lasting effect that persists for days to weeks after interruption of a chronic treatment (Cotzias *et al*. 1967; reviewed by Nutt 2000). After 5 years of treatment, more than 50% of patients receiving levodopa show declining benefit and develop undesired effects such as motor fluctuations and dyskinesias (reviewed by Obeso *et al*. 2000). It has also been suggested that the drug might modify the natural history of PD (for review see Fahn 1996; Murer *et al*. 1999). A better understanding of the long-lasting effects of the drug *in vivo* is therefore necessary.

One of the most frequently studied animal models of PD are rats with partial unilateral lesions of the nigrostriatal pathway induced by injection of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle (Murer *et al*. 1998; Datla *et al*. 2001; reviewed by Deumens *et al*. 2002). With this model, we have shown that a 6-month treatment with doses of levodopa equivalent to those used in clinical practice increases the expression of markers of dopaminergic nerve terminals in the striatum, suggesting that the remaining

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Abbreviations used: bFGF, basic fibroblast growth factor; CAM, calmodulin; cDNA, copy DNA; CFU, colony forming unit; cRNA, copy RNA; DA, dopamine; DAT, DA transporter; DL, dorsolateral; DM, dorsomedial; DOPAC, 3,4-dihydroxylphenylacetic acid; GPX4, glutathione peroxidase 4; GST-7, glutathione S-transferase subunit 7 pi; MAPKK, mitogen-activated protein kinase kinase; MBP, myelin basic protein; mRNA, messenger RNA; NBT/BCIP, nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate; 6-OHDA, 6-hydroxydopamine; PBS, phosphate-buffered saline; PD, Parkinson's disease; PTN, pleiotrophin; ras-GRF, guanine nucleotide release/exchange factor; SSH, suppressive subtractive hybridization; TGFβ, transforming growth factor β; VL, ventrolateral; VM, ventromedial.

dopaminergic nerve fibers in these animals may be capable of sprouting (Murer *et al*. 1998). Similar results have been reported by others (Datla *et al*. 2001).

To identify factors that might contribute to levodopa-induced plasticity in the striatum of 6-OHDA-lesioned rats, we now compare gene expression in this structure in partially lesioned rats treated chronically (6 months) with levodopa or with vehicle alone. We used the technique of suppressive subtractive hybridization (SSH) to construct a cDNA library that was enriched, by selective amplification, in sequences preferentially expressed in levodopa-treated animals. The transcripts in each library were then analyzed by hybridization to a commercially available macroarray (Atlas Rat 1.2 array, Clontech, Palo Alto, CA, USA) of cDNAs corresponding to 1176 rat genes representing the major cell functions.

In the SSH cDNA library constructed from the striata of levodopa-treated 6-OHDA-lesioned rats, we identified transcripts for growth factors, molecules implicated in the remodeling of neural circuitry, elements of intracellular signaling cascades that can be influenced by dopamine (DA) receptors or growth factors and antioxidant enzymes. The differential expression of three particularly interesting genes, pleiotrophin (PTN), a heparin-binding growth factor, myelin basic protein (MBP), and calmodulin (CAM) was confirmed in an independent group of levodopa-treated rats by *in situ* hybridization, and found to be associated with an increase in the levels of the DA transporter (DAT), a marker of dopaminergic innervation.

Materials and methods

Animals

Female Wistar rats (Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina) weighing approximately 200 g at the beginning of the experiments were housed two per cage, with free access to food and liquid, under controlled temperature and a 12-h light/dark cycle. All animal procedures were done in compliance with the Declaration of Helsinki and the NIH Guide for the Care and Use of Laboratory Animals.

Surgery, treatment, and behavior

Partial unilateral lesions of the nigrostriatal pathway were made as previously described (Dziewczapolski *et al*. 1997; Murer *et al*. 1998). Briefly, surgery was performed under deep anesthesia [ketamine/xylazine; 40/2 mg/kg intraperitoneally (i.p.)]. Animals, pretreated with desipramine (25 mg/kg, i.p.) to prevent uptake by noradrenergic neurons, received a stereotaxic injection of 4 μg/2 μL of 6-OHDA (free base, Sigma, St Louis, MO, USA) in distilled water containing 0.1% of ascorbic acid, at the rate of 0.5 μL/min, in the left medial forebrain bundle, 2.8 mm posterior and 2 mm lateral to the bregma and 8.6 mm ventral to the dura (Paxinos and Watson 1986). To confirm the presence of a partial lesion of the nigrostriatal system, contralateral and ipsilateral turning behavior, a classical measure of unilateral dopaminergic denervation of the striatum in rat models (Deumens *et al*. 2002), was recorded in response to challenges with amphetamine (3 mg/kg i.p.) and apomorphine [0.25 mg/kg subcutaneously (s.c)], 2 and 3 weeks, respectively, after lesioning. We included in the experimental protocol those rats making more than 100 ipsilateral turns/h with amphetamine and less than 100 contralateral turns/h with apomorphine.

Levodopa and vehicle treatment began 2 months after lesioning. Commercially available levodopa/carbidopa (170/17 mg/kg/day; Lebocar, Pfizer-Pharmacia, Argentina) was dissolved in tap water, filtered, and made available to rats in light protected bottles. This was their only source of fluid, and was prepared three times a week. The concentration of the levodopa solution after 3 days in the bottles was 90% of the initial value, determined by high-performance liquid chromatography (HPLC; not shown). To keep the doses constant during the 6 months of treatment, the drug concentration was readjusted to the mean weight of the animals and the volume of liquid they drank. Levodopa-treated animals drank significantly less than untreated animals (29.95 ± 3.04 and 34.45 ± 3.82 mL/rat/day, respectively, $p < 0.05$), but the weight of the two groups of animals was similar at the end of treatment (285.1 ± 8 and 294.6 ± 11 g/animal, respectively). The treatment resulted in plasma dopa levels of 1.5 ± 0.3 μg/mL (mean ± SEM), similar to values found in levodopa-treated parkinsonian patients (Högl *et al*. 1998). It also caused a twofold increase in DA turnover in the denervated striatum (DOPAC/DA), determined by HPLC at the end of the dark cycle in a independent group of rats (Rubinstein *et al*. 1997; Högl *et al*. 1998).

A series of behavioral tests were performed during the first and the last month of levodopa treatment 3 h after the lights were turned off and one hour before the end of the dark cycle when the animals were active and drinking the levodopa solution. To evaluate the degree of akinesia of the contralateral forepaw, we performed the stepping (Olsson *et al*. 1995) and cylinder tests (Lundblad *et al*. 2002). In addition, we used an abnormal involuntary movement scale (Lee *et al*. 2000; Delfino *et al*. 2004) and measured spontaneous circling behavior and horizontal locomotor activity over a 24-h period.

Striatal cDNA library enriched in differentially expressed transcripts

The technique of SSH (Diatchenko *et al*. 1996), based on the selective PCR amplification of differentially expressed genes, was used to construct a cDNA library enriched in transcripts expressed in the striatum of 6-OHDA-lesioned rats treated with levodopa (SSH cDNA). A commercially available kit (PCR-Select cDNA subtraction kit, Clontech) was used according to the manufacturer's instructions.

After exposure to CO_2 until loss of consciousness, the animals were killed by decapitation and the brains quickly removed. Striata ipsilateral to the lesions were dissected, pooled by group (seven levodopa, six vehicle) and homogenized by sonication. Polyadenylated mRNA was extracted using commercial methods (RNeasy and Oligotex, Qiagen, Valencia, CA, USA), and double-stranded cDNA were reverse-transcribed from 2 μg of the mRNA. For SSH enrichment of the cDNA from levodopa-treated rats, two different adaptors were ligated to the 5′ end of aliquots of the cDNA. Each aliquot was then hybridized with an excess of adaptor-free cDNA from vehicle-treated rats, leaving single-stranded the adaptor-ligated transcripts which found no complementary strand with which to

hybridize. Subsequent hybridization of the two subtracted aliquots together then permitted these unique transcripts to form double-stranded cDNAs with two different adaptors, which were used for selective amplification of these differentially expressed cDNA by PCR amplifcation with primers corresponding to the adaptor sequences. In this way, all the different cDNAs were amplified together. A second PCR amplification, with a different set of primers, was then performed to enhance selectivity.

Characterization of the SSH levodopa cDNA library

After PCR amplification, the SSH cDNA (150 ng) were inserted into a cloning vector (pGEM-T-easy, Promega, Charbonnières, France), transfected into XL1-Blue MR bacteria (Stratagene, La Jolla, CA, USA), which were then plated on 10 Petri dishes (diameter, 130 mm) and incubated overnight at 37°C. The plated library was replicated on filter lifts (Hybond-N+, Amersham Life Science, Orsay, France), the bacteria lysed and their DNA fixed to the filters for analysis. Standard procedures were used at all stages.

To determine the number of colony forming units (CFU), the filter replicas were hybridized with a non-specific [^{32}P]-labeled probe corresponding to the vector sequence synthesized by random-priming (RadPrime DNA Labeling System, Life Technologies, Rockville, MD, USA), according to the manufacturer's instructions.

The efficacy of the SSH procedure was estimated by determining the percentage of transcripts in the SSH cDNA library that were also found in the vehicle cDNA. The SSH cDNA library replicas were hybridized with a [^{32}P]-labeled probe (10^6 cpm/filter) synthesized by random-priming using a large amount (500 ng) of total striatal vehicle cDNA as the template, to ensure adequate representation of the individual cDNA transcripts present.

To estimate how frequently a given cDNA was present in the SSH library and how many different transcripts were represented, the filter replicas were hybridized with a [^{32}P]-labeled probe synthesized by random-priming of template DNA consisting of pooled cDNAs recovered from 18 randomly selected colonies with cDNA inserts of different sizes. The average number of clones containing each of the 18 cDNAs was calculated, and the total number of different cDNAs extrapolated to the library as a whole.

The number of labeled clones in each of the above experiments was determined after exposure for 7 days at −80°C to X-Omat AR film (Kodak, Rochester, NY, USA). Between hybridizations with each of the probes, the filters were stripped of the radioactive label, then re-exposed to film for 7 days to ensure that there was no residual labeling.

Macroarray analysis of SSH cDNA

The Atlas 1.2 cDNA rat expression array (Clontech), containing 1176 cDNAs corresponding to known genes representing the major cell functions, was used to identify transcripts enriched in the SSH cDNA. [^{32}P]-labeled probes were random-primed (RadPrime DNA Labeling System, Life Technologies) from 200 ng of the SSH cDNA (1×10^7 cpm) and hybridized to the macroarray filters according to the manufacturer's instructions. The procedure was performed twice with new membranes and freshly synthesized probes. The filters were exposed to Fujifilm MP imaging plates and analyzed by a phosphoimaging system (FLA2000, Fuji, Tokyo, Japan), and were also exposed to Kodak X-Omat AR film at − 80°C for 3 and 7 days. Only cDNAs that were labeled both times with SSH cDNA probes (95% concordance) were considered for analysis.

Immunoautoradiographic analysis of the dopamine transporter

The immunoautoradiographic detection of DAT was performed on striatal sections at the same anatomical level as for *in situ* hybridization, as previously described (Murer *et al*. 1998). Briefly, slides were incubated with primary antibodies (rabbit anti-DAT, kindly provided by B. Giros, Paris, France) for 24 h at 4°C, washed in phosphate-buffered saline (PBS), incubated for 2 h with an [^{35}S]-labeled secondary antibody (Amersham, Arlington Heights, IL, USA), washed, dried, and exposed to Hyperfilm Beta-max (Amersham) at 4°C for 7 days. Films were scanned at high resolution (300 dpi) and gray intensity was measured and analyzed with Scion Image software (Scion Corporation, Frederick, MD, USA). Data is expressed as relative optical densities with respect to an internal curve obtained by a spotted [^{35}S]-labeled scale.

In situ hybridization

In situ hybridization was used to confirm the differential expression of representative transcripts identified by macroarray analysis. To generate digoxigenin-labeled cRNA probes, we amplified transcripts corresponding to the sequences spotted on the macroarray by PCR using primers provided by Clontech. The resulting cDNAs (200–300 bp) were purified (QIAquick PCR Purification Kit; Qiagen, Valencia, CA, USA) and cloned into a plasmid vector (pGEM-T easy, Promega), to allow transcription of sense and antisense RNA probes (DIG RNA Labeling Kit, Roche, Mannheim, Germany), according to the manufacturer's instructions, from 1 μg of linearized plasmid using the T7 and SP6 RNA polymerases.

The probes were hybridized to serial slide-mounted frozen coronal brain sections (16 μm), from 1.6 mm anterior to 1.4 mm posterior to the bregma (Paxinos and Watson 1986), from independent groups of lesioned rats treated for 6 months with levodopa/carbidopa ($n = 5$) or vehicle ($n = 4$). The sections were air-dried and post-fixed for 30 min in 4% paraformaldehyde, washed in 2 × saline–sodium citrate (SSC), 0.1 M PBS and acetylated for 10 min with 0.25% acetic anhydride in 0.1 M triethanolamine. The tissue was washed again in 2 × SSC, 0.1 M PBS, dehydrated, and air-dried. Each section was covered with 40 μL of hybridization buffer (50% formamide, 1 × Denhardt's, 0.025% yeast tRNA, 10% dextran sulfate, 0.3 M NaCl, 20 mM Tris, 5 mM EDTA, 10 mM NaH_2PO) containing 10–15 ng of probe per section and incubated for 40 h at 50°C in humid chambers. After hybridization, the sections were washed in 2 × SSC and treated with RNAse A (100 mg/mL) in 2 × SSC at 37°C for 30 min, then washed four times in 2 × SSC at 37°C. The hybrids were detected by immunohistochemistry with an antibody against digoxigenin. The sections were then washed twice in buffer 1 (0.1 M Tris–HCl pH 7.5/0.15 M NaCl), blocked with 2% normal goat serum/0.1% Triton X-100 in buffer 1 and incubated with an alkaline phosphate-conjugated antidigoxigenin antibody (dilution 1/2000; Roche, Germany) for 2 h at room temperature in a humid chamber, washed in buffer 1 then 2 (0.1 M Tris/0.1 M NaCl/0.05 M $MgCl_2$, pH 9.5), then incubated overnight with the substrate NBT/BCIP (2% final dilution; Roche)/1 mM levamisole (ICN, East Hills, NY, USA) in buffer 2 at 37°C in dark humid chambers. Staining was stopped

with buffer to neutralize the pH (0.01 M Tris/1 mM EDTA, pH 8.1), the sections were counterstained with methyl green (Sigma), washed with distilled water and mounted (Aquamount Gurr, BDH, Poole, UK). High-resolution microphotographs were taken from the dorsolateral (DL), dorsomedial (DM), ventrolateral (VL), and ventromedial (VM) striatal regions, as defined previously (Murer *et al*. 1998). The number of labeled cells was counted in four 0.25 mm^2 subregions of each striatum, one in each subregion, in the coronal plane 0.7–1 mm anterior to bregma (Paxinos and Watson 1986).

Statistical analysis

DAT radioimmunolabeling and the number of labeled cells obtained by *in situ* hybridization in the denervated striatum were analyzed by two way ANOVA, with treatment (levodopa vs. vehicle) and regions (DL, DM, VL, and VM) as factors where appropriate. Post hoc comparisons were made by the least significant difference test (LSD), with the level of significance set at $p < 0.05$.

Results

Levodopa-induced behaviors in rats with unilateral 6-OHDA lesions

A series of non-pharmacological tests were used to evaluate behavioral differences and/or functional recovery during levodopa treatment. There was an increase in spontaneous locomotor activity during the period in which the rats received levodopa in their drinking water (data not shown), probably a consequence of greater DA stimulation in these animals (see Methods). Abnormal involuntary movements were not observed in either group. There were also no differences in performance in the stepping or cylinder tests, or in spontaneous circling behavior. The slow continuous mode of administration of levodopa in our animals may explain the absence of rotational behavior and dyskinesias, which are usually seen after acute administration of the drug, and dopaminergic denervation was not severe enough in our animals for supersensitive behaviors to develop (Winkler *et al*. 2002).

Characterization of the SSH levodopa cDNA library

The plated SSH levodopa cDNA library consisted of 15 000 clones, determined by counting colonies labeled with a radioactive probe corresponding to the cloning vector. More than 90% of the colonies contained transcripts that were preferentially expressed in the striatum of levodopa-treated animals; only about 1000 colonies (7–8%) were also labeled by a probe corresponding to cDNAs from vehicle-treated animals. Approximately 1400 colonies were labeled with a probe corresponding to cDNAs from 18 different colonies, suggesting that each of the cDNAs was present in an average of 77 clones each in the 1400 colonies. By extrapolation, there were approximately 200 different transcripts in the 15 000 colonies of the library as a whole.

Macroarray analysis of SSH levodopa cDNA

To identify some of the genes differentially expressed in the striatum of levodopa-treated rats with 6-OHDA-induced lesions of the nigrostriatal pathway, we prepared probes from the SSH cDNA that we hybridized to the Atlas 1.2 rat expression macroarray (Clontech), which contains 1176 cDNAs representing genes covering the major cell functions. The procedure was then repeated with a new macroarray filter and freshly synthesized probes. Only cDNAs labeled in both experiments (95% of labeled clones) were considered to be positive. Due to the SSH procedure that includes a nested PCR amplification that tends to equalize the representation of differentially expressed transcripts in the SSH cDNA, the intensity of labeling was not taken into account, only the identity of the labeled sequences.

Within the limits of the sensitivity of the detection system, 72 genes were identified that could be classified into several categories (Table 1). In addition to a number of genes implicated in basic metabolic processes, mitochondrial activity or protein synthesis, there were two categories of genes that seemed particularly likely to be involved in compensatory plasticity of the nigrostriatal system in levodopa-treated rats: the extracellular signals and receptors, and the metalloproteinases or their inhibitors. Also of great interest was the presence of two myelin-related markers among the differentially expressed cDNA. A number of intracellular signalling molecules, some of which have been reported to participate in neurite extension, were also identified in our screen.

Effects of levodopa on dopaminergic axon terminals in the striatum of rats with unilateral 6-OHDA lesions

To determine whether levodopa treatment induced changes in the dopaminergic innervation of the striatum in 6-OHDA-lesioned rats, we performed an immunoautoradiographic study with an antibody against the DAT, to visualize the dopaminergic fibers in an independent group of levodopa- and vehicle-treated 6-OHDA-lesioned rats. As shown in Fig. 1, on a coronal brain section, 0.7–1 mm anterior to the bregma, the level of DAT immunoreactivity was higher in the striatum ipsilateral to the lesion in rats treated with levodopa than in rats treated with vehicle. This suggests that levodopa may increase the dopaminergic innervation of the striatum in our model, as it was previously shown (Murer *et al*. 1998).

PTN, MBP, and CAM expression in the striatum of levodopa- and vehicle-treated rats with partial unilateral lesions of the nigrostriatal pathway

To confirm that the transcripts were indeed upregulated in the denervated striatum of levodopa-treated animals, we chose a representative transcript of the three most promising categories for study by *in situ* hybridization with digoxigenin-labeled cRNA sense and antisense probes on tissue sections of brains from the same animals in which dopaminergic

Table 1 Levodopa-related gene expression in the striatum of rats with partial unilateral 6-OHDA lesions in the medial forebrain bundle

Category	Name	GeneBank
Growth factors, receptors, and other extracellular signals	Gastric inhibitory polypeptide (GIP)	L08831
	TGF-β receptor II (TGFβ-R2)	L09653
	Pleiotrophin (PTN)	M55601
	Basic fibroblast growth factor (bFGF)	M22427
	Insulin receptor	M29014
	Neurotensin receptor type 2	X97121
	Prolactine-like protein A	M13750
	Substance P	M15191
Metalloproteinases and inhibitors	Carboxypeptidase E (CPE)	M31602
	Tissue inhibitor of metalloproteinase 2	L31884
Myelin-related proteins	**Myelin basic protein (MBP)**	M25889
	Myelin proteolipid protein (PLP)	M11185
Intracellular signaling factors	**Calmodulin (CAM)**	X13817
	Ras-GRF	X67241
	MAP kinase kinase 1/MEK1	Z16415
	Phosphatase 2 A	X16043
	14-kDa phospholipase A2 (PLA2)	U07798
	14-3-3 protein ζ/δ	D17615
	Inositol 1,4,5-triphosphate 3-kinase	X56917
	Male germ cell-associated kinase	M35862
	Chloride channel RCL1	D13985
	Ras-related protein RAB14	M83680
	Rgs4; regulator of G protein signaling 4 (RGP4)	U27767
	G protein-coupled receptor, putative, (GPR6)	U12006
Metabolic enzymes	Soluble superoxide dismutase 1 (SOD1)	Y00404
	Carbonic anhydrase 4	S68245
	Carbonic anhydrase 5	U12268
	$NADP^+$ alcohol dehydrogenase	D10854
	Endothelial NOS	U02534
	Glutathione peroxidase 4 (GPX4)	X82679
	Glutathione S-transferase subunit 7 pi	X02904
	Aldolase C	X06984
Proteasomal proteins	Proteasome component C8	M58593
	Proteasome ι subunit	D10755
	Proteasome β subunit	L17127
	Proteasome subunit C5	X52783
Ribosomal proteins	60S ribosomal protein L44 (RPL44)	M19635
	40S ribosomal protein S12 (RPS12)	M18547
	X-linked 40S ribosomal protein S4 (RPS4X)	X14210
	Ribosomal protein L11	X62146
	Ribosomal protein L13	X78327
	40S ribosomal protein S19 (RPS19)	X51707
	60S ribosomal protein L21 (RPL21)	M27905
	60S ribosomal protein L19 (RPL19)	J02650
	40S ribosomal protein S11 (RPS11)	K03250
	40S ribosomal protein S17 (RPS17)	K02933
	40S ribosomal protein S3A (RPS3A)	M84716
Transporters and ion channels	Fibroblast ADP/ATP carrier protein	D12771
	Sodium-dependent glutamate/aspartate transporter 1	S59158
	ATP synthase lipid-binding protein P1;	D13123
	Voltage-gated potassium channel protein 3.2 (KV3.2)	M84203
	Sodium channel β 1 subunit	M91808
	Sodium/potassium-transporting ATPase β 1 subunit	J02701
	Sodium/potassium-transporting ATPase α 2 subunit	M14512
	Apolipoprotein D	X55572

Table 1 continued

Category	Name	GeneBank
Cysteine proteases	Cathepsin H	M36320
	Cathepsin S	L03201
	Cathepsin L	Y00697
	Cathepsin B	X82396
Energy metabolism	Cytochrome c oxidase polypeptide Vb (COX5B)	D10952
	Sertoli cell cytochrome *c* oxidasepolypeptide I (COX1)	S79304
	Cytochrome *c* oxidase subunit IV (COX4)	X14209
	Liver/heart cytochrome c oxidase polypeptide VIIa (COX7A)	X54080
	Mitochondrial ATP synthase D subunit	D10021
	Mitochondrial ATP synthase β subunit	M19044
	Mitochondrial ATP synthase B subunit	M35052
	Mitochondrial H^+ transporting ATP synthase F1 complex α	X56133
Other	Heat shock 90-kDa protein β (HSP-90β)	S45392
	Eukaryotic translation initiation factor 5	L11651
	β2-microglobulin	X16956
	Nucleoside diphosphate kinase B	M91597
	B-cell receptor-associated protein 32	M61219

In bold are those transcripts also shown increased by *in situ* hybridization. GeneBank: access numbers to the GeneBank database.

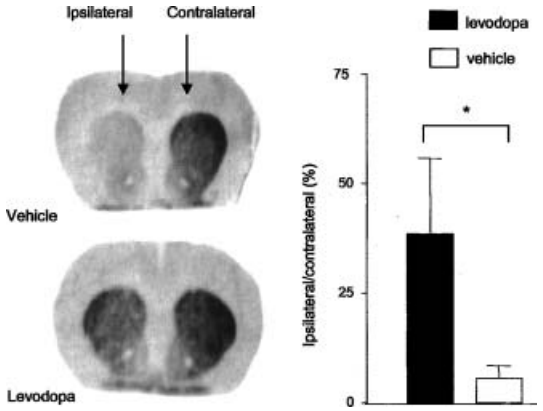

Fig. 1 Expression of the dopamine transporter in the striatum of vehicle- and levodopa-treated rats with unilateral 6-OHDA lesions of the medial forebrain bundle. Left: The levels of expression of the dopamine transporter (DAT) were evaluated by autoradiography on coronal brain sections. The hemispheres ipsi- and contralateral to the lesion are indicated by arrows. Right: DAT immunoreactivity was quantified by measuring grey levels in the DL, DM, VL, and VM striatum. DAT levels ipsilateral to the lesion are represented as the percentage of levels in the striatum contralateral to the lesion (mean ± SEM). A two-way ANOVA (treatment and striatal regions as factors) revealed a treatment effect [$F_{1,28} = 10.57$], $^*p = 0.003$; ■, levodopa; □, vehicle. No significant effects of region or interaction were observed.

innervation was evaluated: the heparin-binding growth factor pleiotrophin, myelin basic protein and the calcium binding protein calmodulin.

In situ hybridization was performed at the same anatomical level as the DAT immunoautography shown above (Fig. 1). The digoxigenin-labeled sense cRNA probes, the sequences of which were identical to PTN, MBP, and CAM mRNA, produced only a low level of background staining (Fig. 2), whereas the antisense cRNA probes hybridized specifically with transcripts in the striatum of both levodopa- and vehicle-treated rats (Fig. 2).

The results were quantified by counting the number of labeled cells in subregions of the striatum (DL, VL, DM, VM) ipsilateral and contralateral to the lesion (Table 2). Levodopa treatment increased the number of cells labeled by each of the probes in the striatum ipsilateral to the lesion, but to different degrees (Table 2a). A post-hoc analysis was undertaken in the subregions to determine whether there was a common regional pattern of overexpression for the three transcripts. The subregions in which statistically significant increases were observed were different for each of the three transcripts (Table 2a). In general, the strongest effects were observed in the dorsal and ventromedial striatum. The greatest increase was observed for PTN-labeled cells, which increased significantly in number in the DL, DM, and VM. Significant increases in the number of MBP- and CAM-expressing cells were observed in the DL and DM, respectively. In the striatum contralateral to the lesion, no significant differences in the expression of PTN, MBP or CAM were observed (Table 2b).

PTN and CAM were localized in neurons, according to morphological criteria (shape of cell body and size of nucleus). MBP was mainly localized in glial cells, but MBP labeling could also be observed in some neurons.

Discussion

Since the initial description of SSH (Diatchenko *et al*. 1996), this PCR-based technique has become widely used for the

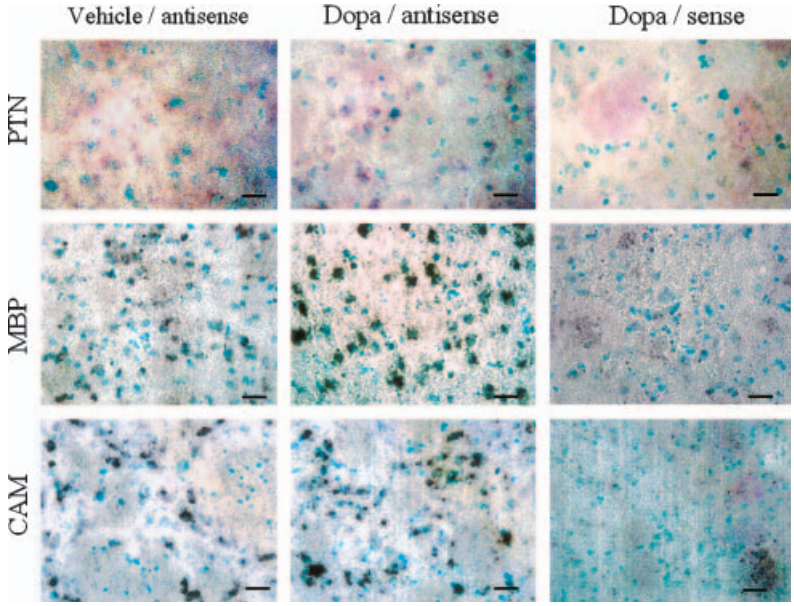

Fig. 2 PTN, MBP and CAM expression in the DL striatum of rats with unilateral 6-OHDA-induced lesions treated for 6 months with vehicle or levodopa. Representative photomicrographs of *in situ* hybridization with digoxigenin-labeled antisense and sense cRNA probes, revealed by immunocytochemistry with NBT/BCIP as the chromagen (dark deposits). Scale bars = 50 μm.

isolation of differentially expressed transcripts. Its efficacy is increased when associated with the analysis of expression arrays (Beck *et al*. 2001; Swearingen *et al*. 2003). We have used such a combination to identify factors that might contribute to a levodopa-induced increase in dopaminergic innervation in the striatum after denervation, using a well-studied model of Parkinson's disease, rats with partial unilateral lesions of medial forebrain bundle induced by 6-OHDA (reviewed by Deumens *et al*. 2002).

The choice of SSH, rather than a differential screen based on levels of expression, was based on the hypothesis that major genes involved in levodopa-induced nigrostriatal plasticity would not be expressed in the absence of the drug. Alternatively, levodopa would have enhanced the expression of transcripts that mediate the spontaneous recovery of the damaged nigrostriatal system (Blanchard *et al*. 1996; Finkelstein *et al*. 2000), which would presumably be expressed at very low levels in vehicle-treated rats. We therefore chose a technique intended to enhance the representation of genes expressed in close to an all or nothing manner in the experimental conditions for which the cDNA library was constructed. This was expected to result in the identification of a small number of relevant transcripts, rather than a large number based on a preliminary analysis of their levels of expression. Furthermore, as we could not predict the level at which such genes would be expressed, the SSH technique had the second advantage of increasing the representation of such transcripts in the final library increasing the probability that genes expressed differentially but at low levels would be detected.

In accordance with our expectations, characterization of our SSH levodopa cDNA library showed that a relatively small number of transcripts (approximately 200) were selectively amplified. Cross-hybridization of the SSH levodopa library with cDNA from vehicle-treated controls attested to the efficacy of the selection procedure, as only 7–8% of clones of the plated library expressed sequences also found among the vehicle cDNA. The selection process intended to enrich the levodopa cDNA library in transcripts specific to the striatum of levodopa-treated 6-OHDA-lesioned rats was therefore successful.

A screen of the Atlas 1.2 rat brain expression array permitted identification of 72 of the transcripts preferentially expressed in the striatum of levodopa-treated animals, many of which have known functions related to neurite outgrowth. Although transcripts in the SSH levodopa cDNA that were not represented on the Atlas 1.2 macroarray still remain to be identified, those already identified have provided interesting clues to the molecular mechanism of levodopa-induced plasticity.

Growth factors and enzymes involved in remodeling the extracellular matrix are among the most evident candidates. Pleiotrophin, bFGF and TGF-β receptor have previously been associated with neurite outgrowth, not only during development (Wanaka *et al*. 1993; Poulsen *et al*. 1994; Kinnunen *et al*. 1998; Deuel *et al*. 2002), but also in adult brain following experimental lesions (Haynes 1988; Yeh *et al*. 1998). Metalloproteinases and their inhibitors are also involved in circuit remodeling through their actions on the extracellular matrix (Vaillant *et al*. 1999). The two myelin-related proteins, MBP and proteolipid protein, are perhaps the most intriguing of the transcripts identified. The fact that these factors, which play important roles in myelination, were both identified among SSH selected transcripts suggests that they were indeed selected because they are upregulated in the denervated striatum of 6-OHDA-lesioned rats following

Table 2 Number of PTN-, MBP-, and CAM-positive cells labeled by *in situ* hybridization in the striatum ipsilateral (A) and contralateral (B) to a 6-OHDA-lesion in rats treated for 6 months with vehicle ($n = 4$) or levodopa ($n = 5$)

Ipsilateral striatum	Vehicle (mean ± SEM)	Levodopa (mean ± SEM)	Levodopa/ Vehicle (%)	*p*-value
A: Ipsilateral (lesioned) striatum				
PTN				
Treatment				***0.001***
Region				***0.001***
Interaction				*NS*
DL	7.25 ± 2.47	24.40 ± 3.24	337%	0.033
DM	9.75 ± 3.12	31.20 ± 5.32	328%	0.015
VL	3.70 ± 2.09	9.60 ± 2.62	259%	0.531
VM	4.50 ± 2.63	17.60 ± 4.91	391%	0.037
MBP				
Treatment				***0.048***
Region				*NS*
Interaction				*NS*
DL	12.40 ± 2.42	20.70 ± 3.88	167%	0.033
DM	18.00 ± 3.34	21.50 ± 4.29	119%	0.488
VL	17.25 ± 3.57	16.80 ± 3.68	97%	1.000
VM	11.75 ± 1.89	17.40 ± 3.17	148%	0.242
CAM				
Treatment				***0.006***
Region				*NS*
Interaction				*NS*
DL	17.00 ± 2.38	23.00 ± 2.88	135%	0.132
DM	11.00 ± 1.99	19.00 ± 3.56	173%	0.039
VL	17.50 ± 3.59	18.20 ± 3.85	104%	0.729
VM	15.00 ± 3.29	20.80 ± 1.16	139%	0.079
B: Contralateral (unlesioned) striatum				
PTN				
Treatment				*NS*
Region				
Interaction				
DL	12.00 ± 7.54	15.80 ± 5.52	132%	NS
DM	ND	ND	–	–
VL	ND	ND	–	–
VM	ND	ND	–	–
MBP				
Treatment				*NS*
Region				*NS*
Interaction				*NS*
DL	14.50 ± 1.18	17.30 ± 1.71	119%	NS
DM	17.50 ± 4.02	17.00 ± 3.94	97%	NS
VL	17.30 ± 3.32	17.80 ± 2.30	102%	NS
VM	19.50 ± 2.52	20.20 ± 1.56	104%	NS
CAM				
Treatment				*NS*
Region				*NS*
Interaction				*NS*
DL	17.00 ± 3.17	20.60 ± 2.97	121%	NS
DM	16.00 ± 1.57	21.20 ± 2.10	134%	NS
VL	19.50 ± 5.55	17.20 ± 2.27	88%	NS
VM	22.30 ± 3.14	24.40 ± 1.60	109%	NS

Cells were counted in four striatal regions: DL, DM, VL, and VM. Data were analyzed by two-way ANOVA (treatment and regions as factors). Ipsilateral striatum: treatment effect [PTN ($F_{1,28} = 14.6$), MBP ($F_{1,27} = 4.25$) and CAM ($F_{1,28} = 8.66$)]; region effect [PTN ($F_{3,28} = 4.47$), MBP ($F_{3,27} = 0.89$) and CAM ($F_{3,28} = 1.64$)]; interaction [PTN ($F_{3,28} = 0.75$), MBP ($F_{3,27} = 0.88$) and CAM ($F_{3,28} = 0.62$)]. Contralateral striatum: treatment effect [PTN ($F_{1,7} = 0.23$), MBP ($F_{1,28} = 0.26$) and CAM ($F_{1,28} = 1.42$)]; region effect [MBP ($F_{3,28} = 0.92$) and CAM ($F_{3,28} = 1.88$)]; interaction [MBP ($F_{3,28} = 0.16$) and CAM ($F_{3,28} = 0.83$)]. *p*-values for ANOVA are in italics. Post-hoc comparisons were performed by LSD test. *p*-values indicating significant differences are in bold. ND, not determined. NS, not statistically significant.

levodopa treatment, and do not represent non-specific background. Some metabolic enzymes as superoxide dismutase 1, glutathione peroxidase 4 (GPX4) and glutathion S-transferase subunit 7 pi (GST-7), could be involved in compensatory protection against oxygen reactive species produced by dopa metabolism (Cohen 2000). Major signal transduction molecules were also identified. The guanine nucleotide release/exchange factor (Ras-GRF), mitogen-activated protein kinase kinase (MAPKK), and CAM have been associated with neuronal plasticity in the CNS (reviewed by Impey *et al*. 1999) and with DA stimulation (Michelhaugh and Gnegy 2000; Gerfen 2003).

Some of the library transcripts have been reported by others to be upregulated in patients with PD or animal models of PD under levodopa treatment. β2-Microglobuline (Mogi *et al*. 1995), bFGF, and TGF-β (a TGF-β receptor was identified in the SSH cDNA library) have been reported to be increased in the striatum of patients with PD undergoing levodopa therapy (reviewed by Nagatsu *et al*. 2000). In addition, levodopa has been found to increase the expression of substance P (Salin *et al.* 1997) in the denervated striatum of 6-OHDA-lesioned rats. Superoxide dismutase 1 (Asanuma *et al*. 1998), GST, GPX (Bensadoun *et al*. 1998; Weingarten and Zhou 2001), bFGF (Otto and Unsicker 1990; Reuss and Unsicker 2000), and TGF- β (Unsicker *et al*. 1996) have also been reported to contribute to trophic support of dopaminergic neurons and to protect against oxidative stress in both *in vivo* and *in vitro* models of PD.

Because PCR-amplified SSH cDNA cannot be used in macroarray analysis to determine levels of gene expression, we validated our selection procedure by *in situ* hybridization on an independent series of 6-OHDA-lesioned, levodopa- and vehicle-treated rats. We focused on three promising transcripts identified in our expression screen: PTN, representative of the growth factors, the myelin-associated protein MBP, and the signal transduction molecule CAM. The results obtained by *in situ* hybridization confirmed that there were significant increases in expression of the three candidate transcripts in the denervated striatum of levodopa-treated rats. The combination of SSH and macroarray analysis therefore enabled us to identify factors, PTN and MBP, which may play important roles in levodopa-induced plasticity of the nigrostriatal system.

Pleiotrophin is a secreted heparin-binding growth factor that is highly expressed during early post-natal brain development (Kinnunen *et al*. 1998) and is upregulated after experimental lesions in the hippocampus of adult animals (Takeda *et al*. 1995; Yeh *et al*. 1998; Poulsen *et al*. 2000). Pleiotrophin enhances neurite outgrowth in cultured neurons and has been shown to be involved in axonal guidance (reviewed by Rauvala and Peng 1997). It has been recently shown to promote neurite outgrowth and the survival of dopaminergic neurons in embryonic mesencephalic cultures (Hida *et al*. 2003).

MBP, like proteolipid protein, also detected in our macroarray analysis, is expressed in oligodendrocytes, the myelinating cells of the CNS (Verity and Campagnoni 1988). These cells have been described to have trophic effects on neurons in the basal forebrain (Dai *et al*. 2003; Wilkins *et al*. 2003), and MBP has been reported to be upregulated like PTN in the denervated hippocampus (Jensen *et al*. 2000) and in peri-infarct areas after ischemia (Gregersen *et al*. 2001). Interestingly, our MBP probe also hybridized to some neurons in the striatum ipsilateral to the lesion. The transcript detected may correspond to MBP itself or perhaps to *golli* (*g*ene of the *oli*godendrocyte *li*neage) variants, which are transcribed from the *golli–mbp* gene complex and include mbp sequences in addition to unique peptide sequences, and are expressed in neurons during neural development (Landry *et al*. 1996). These data suggest that the role of oligodendrocytes and/or neuronal MBP-related transcripts in the plasticity of dopaminergic neurons warrants further investigation, particularly as these neurons are not myelinated (Hattori *et al*. 1973).

In conclusion, our strategy of SSH led to the identification of a small number of genes that appear to be induced by levodopa treatment in rats with partial unilateral lesions of nigrostriatal system and in many cases have demonstrated roles in neuronal plasticity. Three of the genes identified with the expression array, PTN, MBP and CAM, which were examined in more detail by *in situ* hybridization, were clearly upregulated in the denervated striatum of levodopa-treated animals. Both the trophic factor PTN and the myelin-related proteins open particularly interesting and novel perspectives for understanding the mechanism of dopaminergic plasticity in this experimental model. The other genes identified in this screening procedure will hopefully be as informative.

Despite the differences in the mechanisms of denervation and the plastic responses in the model used and PD, which have been discussed elsewhere (Murer *et al*. 1998, 1999; Hirsch 2000), the general features of the biological processes underlying these phenomena in the two conditions might be similar. It must be determined whether the sprouting induced by levodopa in the denervated striatum of the 6-OHDA-treated rats also takes place in patients. Is this plasticity of the nigrostriatal pathway implicated in the deleterious effects of long-term levodopa treatment? Is it responsible for the prolonged benefit observed in patients after withdrawal of the drug, and if so, can it be exploited for therapeutic purposes? The results of the present study have provided new molecular tools with which to approach these questions.

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