Pergamon

PII: S0306-4522(00)00223-2

www.elsevier.com/locate/neuroscience

Neuroscience Vol. 99, No. 4, pp. 643–650, 2000

0306-4522/00 $20.00+0.00

THE INDIRECT BASAL GANGLIA PATHWAY IN DOPAMINE D_2 RECEPTOR-DEFICIENT MICE

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Abstract—Recent pathophysiological models of basal ganglia function in Parkinson's disease predict that specific neurochemical changes in the indirect pathway would follow the lack of stimulation of D_2 dopamine receptors. *Post mortem* studies of the basal ganglia in genetically modified mice lacking functional copies of the D_2 dopamine receptor gene allowed us to test these predictions. When compared with their congenic N_5 wild-type siblings, mice lacking D_2 receptors show an increased expression of enkephalin messenger RNA in the striatum, and an increased activity and expression of cytochrome oxidase I in the subthalamic nucleus, as expected. In addition, D_2 receptor-deficient mice display a reduced expression of glutamate decarboxylase-67 messenger RNA in the globus pallidus, as the basal ganglia model predicts. This reduction contrasts with the lack of change or increase in glutamate decarboxylase-67 messenger RNA expression found in animals depleted of dopamine after lesions of the mesostriatal dopaminergic system. Furthermore, D_2 receptor-deficient mice show a significant decrease in substance P messenger RNA expression in the striatonigral neurons which form the direct pathway. Finally, glutamate decarboxylase-67 messenger RNA expression in the basal ganglia output nuclei was not affected by mutations in the D_2 receptor gene, a fact that could probably be related to the absence of a parkinsonian locomotor phenotype in D_2 receptor-deficient mice.

In summary, these findings provide compelling evidence demonstrating that the lack of endogenous stimulation of D_2 receptors is sufficient to produce subthalamic nucleus hyperactivity, as assessed by cytochrome oxidase I histochemistry and messenger RNA expression, and strongly suggest the existence of interactions between the basal ganglia direct and indirect pathways. © 2000 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: globus pallidus, Parkinson's disease, striatum, subthalamic nucleus, substantia nigra.

The clinical manifestations of Parkinson's disease (PD) result from the degeneration of mesencephalic dopaminergic neurons and the associated decrease of striatal dopamine content. According to current models of PD pathophysiology,[11,44] the lack of stimulation of striatal dopamine receptors will produce specific functional changes in neurons of the basal ganglia direct (striatonigral/striatoentopeduncular) and indirect (striato-pallido-subthalamic) pathways. Gerfen *et al.*[16] have shown, in the rat lesioned unilaterally with 6-hydroxydopamine, a widespread rodent model of PD, that the striatal expression of enkephalin mRNA (a marker of striatopallidal neurons) is augmented, while expression of substance P mRNA (a marker of striatonigral neurons) is diminished, and that these changes can be selectively reversed by D_2- and D_1-type receptor agonists, respectively. Changes of striatal function in parkinsonian animals would lead to hyperactivity of output nuclei neurons,[14,27] presumably as a consequence of two concurrent processes: (i) reduced activity of the inhibitory, D_1-governed, direct pathway; (ii) increased activity of the subthalamic nucleus (STN). Increased STN activity is assumed to result from the hyperactivity of striatopallidal (D_2-governed) neurons and subsequent reduction of the tonic inhibitory influence of the globus pallidus (GP) on the STN[11,28,31] (but see reviews in Refs 10 and 26). Thus, during the last decade, a picture of what seems to be a parkinsonian basal ganglia neurochemical phenotype has arisen, and includes an increase in striatal enkephalin mRNA expression and a decrease in striatal substance P expression,[4,16,19,35] increased STN cytochrome oxidase I (COI) activity, suggesting increased STN neuronal activity,[32,40,41] and increased glutamate decarboxylase-67 (GAD_{67}) mRNA expression in the basal ganglia output nuclei.[20,37,39,42]

Most evidence regarding the contribution of D_1- and D_2-type dopamine receptors in the genesis of the behavioral and neurochemical parkinsonian phenotype has been provided by classic pharmacological approaches. More recently, genetically modified mice provided the opportunity of selectively studying the consequences of the lack of functional copies of specific dopamine receptor genes.[1,5,13,24,33,46] Current concepts of the functional organization of the basal ganglia suggest a critical role for D_2 receptors and the indirect pathway in the pathophysiology of PD.[10,26,36] The main signs of the disease are akinesia, tremor and rigidity. We have recently reported that D_2 receptor-deficient mice exhibit a phenotype that only

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Abbreviations: COI, cytochrome oxidase I; GAD, glutamate decarboxylase; GP, globus pallidus; PD, Parkinson's disease; SSC, standard saline citrate; STN, subthalamic nucleus.

partially resembles the clinical manifestations of PD.[24] Although mice lacking the two functional copies of the D_2 receptor showed a deficit in movement initiation, they have normal movement speed, no postural abnormalities and no tremor.

In order to determine if a selective deficit in D_2 receptor-mediated dopamine actions can lead to the development of the distinguishing neurochemical changes occurring in the basal ganglia indirect pathway in PD, we studied the expression of several neurochemical markers in the basal ganglia of mice lacking one or two functional copies of the D_2 receptor and their wild-type siblings.

EXPERIMENTAL PROCEDURES

D_2 dopamine receptor-deficient mice

The original F_2 hybrid strain (129/Sv × C57BL/6J) of mice containing the mutated D_2 dopamine receptor allele was obtained as described previously.[23] For the experiments described below, we used congenic N_5 mice of both sexes, generated by back-crossing D_2 dopamine receptor +/− mice to wild-type C57BL/6J mice for five generations, as reported.[23,24]

Tissue preparation

Mice were killed 15 weeks after birth, following the guidelines of the NIH Guide for Care and Use of Laboratory Animals. Their brains were quickly removed, frozen in isopentane at −30°C and stored until use at −80°C. Coronal 15-μm-thick tissue sections were cut in a cryostat throughout the mesencephalon, diencephalon and telencephalon, and thaw-mounted on to cold gelatin-coated glass slides. Tissue sections were dehydrated at room temperature for 10 min and stored at −80°C until processing.

Quantitative in situ *hybridization histochemistry*

Oligonucleotide DNA probes (45 oligonucleotides long) were used for substance P and enkephalin *in situ* hybridization, as reported.[35] The probes were 3′ end-labeled by terminal deoxynucleotide transferase with [^{35}S]dATP (1300 Ci/mmol; NEN, Boston, MA, USA) using a DNA tailing kit (NEN). The labeled probes were purified on commercially available columns (NENSORB 20, NEN). The *in situ* hybridization protocol used with the oligoprobes has been published elsewhere.[35] In brief, slide-mounted sections were postfixed for 5 min in 3% paraformaldehyde, incubated for 1 h in pre-hybridization buffer [4 × standard saline citrate (SSC) and 1 × Denhardt's solution] and acetylated for 10 min in 0.25% acetic anhydride/0.1 M triethanolamine. Then, the tissue was treated for 30 min with 1 M Tris–glycine, dehydrated and air dried. Each section was covered with 35 μl of hybridization solution (50% formamide, 1 × Denhardt's, 1% yeast tRNA, 1% sheared salmon sperm DNA, 10% dextran sulfate, 4 × SSC) containing 20 nmol of radiolabeled probe (~400,000 c.p.m.) and incubated for 12–16 h at 40°C in humid chambers. After hybridization, the sections were washed in 1 × SSC for 1 h at room temperature, 1 × SSC for 1 h at 40°C, 0.1 × SSC for 1 h at 40°C, dehydrated and apposed to Kodak X-OMAT AR5 films (Kodak, Integra Bioscience, Eaubonne, France) for seven to 10 days.

^{35}S-Labeled RNA probes were used for GAD_{67} and COI *in situ* hybridization. The GAD_{67} probe was transcribed *in vitro* following the method published by Fontaine *et al.*[15] from a 2.7-kb cDNA sequence corresponding to the 67,000 mol. wt human GAD gene, as reported.[25] The COI probe was transcribed *in vitro* following the same protocol, from an 857-base cDNA sequence containing bases 5393–6250 of the mouse COI gene, flanked by the T7 and SP6 RNA polymerase promoters. In brief, mouse DNA was isolated and the COI sequence amplified by polymerase chain reaction with primers to which the T7 and SP6 promoter sequences were added to the 5′ ends: the primer complementary to the 3′ end of the chosen genomic COI DNA sequence (5′ GCTGATGTAAAGCAAGCTCGT 3′) was preceded by the sequence of the promoter of the T7 RNA polymerase (5′ TGTAATACGACTCACTATAGGGCGA 3′), and the primer coding for the 5′ end of the chosen genomic COI gene sequence (5′ CGGAGCCTGAGCGGGAATA 3′) was preceded by the sequence of the promoter of the SP6 RNA polymerase (5′ ATTTAGGTGACACTATAGAATACT 3′). The experimental protocol for *in situ* hybridization with labeled RNA probes has already been published.[25] In brief, slide-mounted sections were postfixed for 5 min in 3% paraformaldehyde, acetylated in 0.25% acetic anhydride/0.1 mM triethanolamine, treated with 0.1 M Tris–glycine for 30 min and dehydrated. After air-drying, sections were covered with 50 μl of hybridization solution containing 2.5×10^6 c.p.m. of ^{35}S-labeled probe, and incubated for 3.5 h at 50°C in humid chambers. The slides were then washed at 50°C in 50% formamide/2 × SSC, incubated for 30 min at 37°C with RNase A (100 μg/ml in 2 × SSC), rinsed at 50°C in 50% formamide/2 × SSC and left overnight in 2 × SSC at room temperature. Finally, the sections were dehydrated and treated with 300 mM ammonium acetate, delipidated in xylene, rinsed in 100% ethanol and air-dried. Autoradiograms were generated by exposing the slides to X-ray-sensitive films (Hyperfilm β-max, Amersham, Buckinghamshire, UK) for two to seven days at 4°C. The slides were then dipped in Kodak NTB2 emulsion diluted 1:1, air-dried and stored at 4°C for one to four weeks.

The intensity of autoradiographic labeling was quantified by image analysis (Biocom, Les Ulis, France).[35] Gray levels were converted to optical densities by means of internal standard curves (calibrated density step tablet, Kodak). For a given structure, the optical density was determined in both hemispheres from at least three different sections and averaged to obtain a single mean optical density value per animal. Background labeling was estimated from regions lacking the specific signal and subtracted from total labeling. Grain density at the cellular level was quantified by a computer-assisted semiautomatic image analysis system (HISTO200, Biocom), according to the method of Bisconte *et al.*[6] In brief, grain counts were performed under polarized light on emulsion-coated sections stained with hematoxylin. The image analysis system allows a rapid estimation of the number of grains over a user-defined region, i.e. a neuronal profile. A standard curve of optical density as a function of the number of grains was established for each section. To obtain the standard curve, the observer used several grain clusters comprising unambiguous grain numbers. Through the standard curve, the software converted the optical density over a neuron into a number of grains. Histological staining defined the boundaries of cells. However, grain clusters usually overlapped the histological definition of the cell boundaries. Therefore, optical density per cell profile was measured on the area corresponding to the grain clusters overlying a neuron. Once the area corresponding to the grain cluster was delimited, the computer measured the surface area and the optical density over this area, calculated the number of grains and then the grain density (grains per μm^2). Grain density was taken as an estimation of the level of hybridization. Background grain density was determined from a region lacking specific signal and subtracted from total labeling. For each brain, 100 randomly distributed labeled neuronal profiles were analysed in each structure (50 per hemisphere), and averaged to obtain a single mean grain density value per structure per brain. All experiments were run including sections hybridized with the sense probes in order to test the specificity of labeling.

Cytochrome oxidase histochemistry

COI histochemistry was performed according to the protocol of Wong-Riley,[45] as described elsewhere.[40] Slides were incubated for 4 h at 37°C in 0.1 M phosphate buffer (pH 7.4) containing 0.55 g/l of 3,3′-diaminobenzidine (Sigma-Aldrich, Saint Quentin Fallavier, France), 0.33 g/l of horse heart cytochrome *c* (Sigma), 44 g/l of sucrose (Sigma) and 200 μg/ml of catalase (hydrogen peroxidase oxidoreductase; Sigma). Following incubation, slides were rinsed three times in phosphate buffer, dehydrated and coversliped. Measurements of COI histochemical staining intensity were performed with the aid of a computer-assisted semiautomatic image-analysis system (Biocom). The mean relative optical density per pixel was determined by outlining the structure of interest and subtracting the optical density of the background (estimated at the level of the corpus callosum). For each animal, a single value per structure was obtained by averaging measurements from several sections and from both hemispheres.

Statistics

The effect of genotype on the different measurements was determined with one-way ANOVAs followed by Tukey's test. When data did not fit a normal distribution or groups had unequal variances, a Kruskal–Wallis one-way ANOVA on ranks was preferred, followed by Dunn's method for post hoc comparisons. Statistical significance

Table 1. Changes of striatal markers in D_2 dopamine receptor-deficient mice

	+/+	+/−	−/−
Enkephalin mRNA	10.68 ± 0.21	13.72 ± 0.39*	17.67 ± 0.48*†
Substance P mRNA	10.50 ± 0.23	8.77 ± 0.35‡	8.37 ± 0.29‡
GAD_{67} mRNA	16.99 ± 1.51	16.34 ± 1.59	20.78 ± 1.65

Data are the mean ± S.E.M. relative optical density of nine to 11 mice per group. *$P < 0.05$ vs +/+ and †$P < 0.05$ vs −/+, Tukey's test after significant one-way ANOVA ($F_{2,26} = 98.1$, $P < 0.001$). ‡$P < 0.05$ vs +/+, Tukey's test after significant one-way ANOVA ($F_{2,24} = 16.2$, $P < 0.001$).

Table 2. Glutamate decarboxylase-67 mRNA expression in the globus pallidus, basal ganglia output nuclei and cerebral cortex in D_2 dopamine receptor-deficient mice

	+/+	+/−	−/−
Globus pallidus	0.92 ± 0.06	0.70 ± 0.10	0.65 ± 0.04*
Entopeduncular nucleus	0.59 ± 0.06	0.66 ± 0.07	0.58 ± 0.02
Substantia nigra pars reticulata	0.59 ± 0.07	0.59 ± 0.05	0.65 ± 0.06
Parietal cortex	0.97 ± 0.06	0.85 ± 0.11	0.90 ± 0.04

Data are the mean ± S.E.M. grain density (grains/μm^2) of five to six mice per group. *$P < 0.05$ vs +/+, Tukey's test after significant one-way ANOVA ($F_{2,13} = 3.90$, $P = 0.047$).

was set at $P < 0.05$. Since no effect of sex was observed in any of the variables studied, data from male and female mice were grouped.

RESULTS

Striatum

The striatal neurochemical phenotype of D_2 dopamine receptor-deficient mice closely resembles that of animals chronically depleted of dopamine after 6-hydroxydopamine- or methyl-phenyl-tetrahydropyridine-induced lesions (Table 1, Fig. 1). On the one hand, enkephalin mRNA expression (a marker of the function of striatopallidal neurons) was increased by 29% in D_2 dopamine receptor +/− and 65% in D_2 dopamine receptor −/− mice compared to controls. On the other hand, substance P mRNA expression (a marker of striatonigral function) was unexpectedly found to be significantly decreased to 83% and 80% of the control values in D_2 dopamine receptor +/− and −/− mice, respectively. No significant change in the expression of GAD_{67} mRNA was observed in this brain area.

Globus pallidus

The expression of GAD_{67} mRNA was significantly reduced in D_2 dopamine receptor −/− mice to levels that were

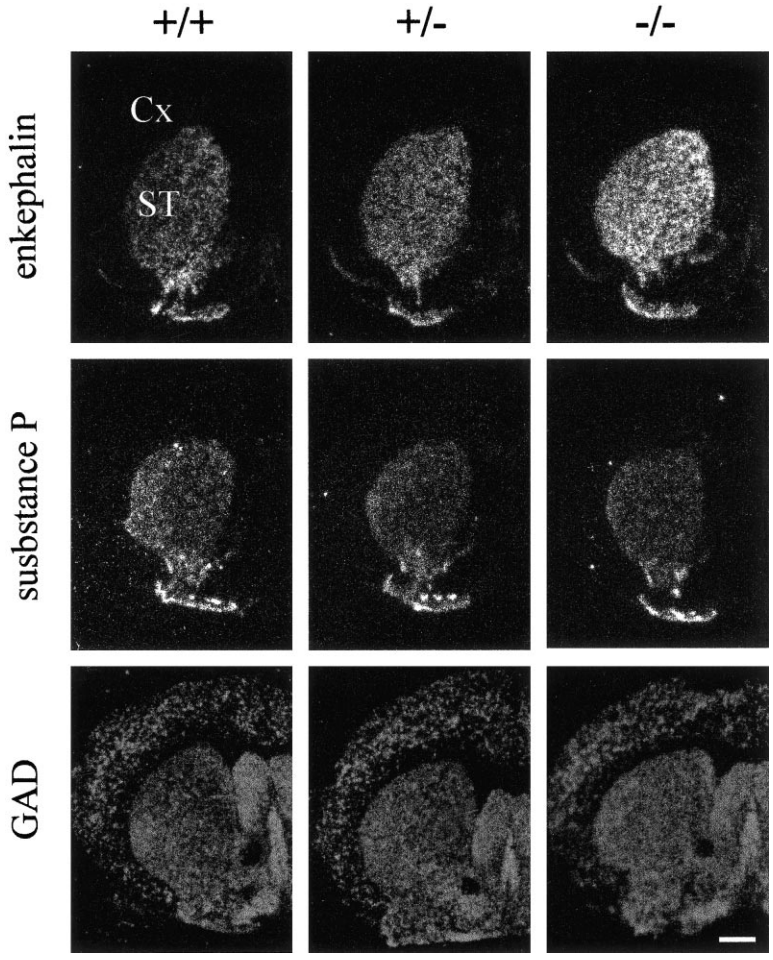

Fig. 1. Neurochemical changes occurring in the striatum of D_2 dopamine receptor-deficient mice. Film autoradiograms obtained from coronal sections hybridized with ^{35}S-labeled probes directed against enkephalin mRNA (upper row), substance P mRNA (central row) and GAD_{67} mRNA (lower row). Note the increased expression of enkephalin mRNA and the reduced expression of substance P mRNA observed in mice lacking one or both functional copies of the D_2 receptor gene when compared with their congenic N_5 wild type-siblings. No change in GAD_{67} mRNA expression was observed in the striatum. Cx, cortex; ST, striatum. Scale bar = 1 mm.

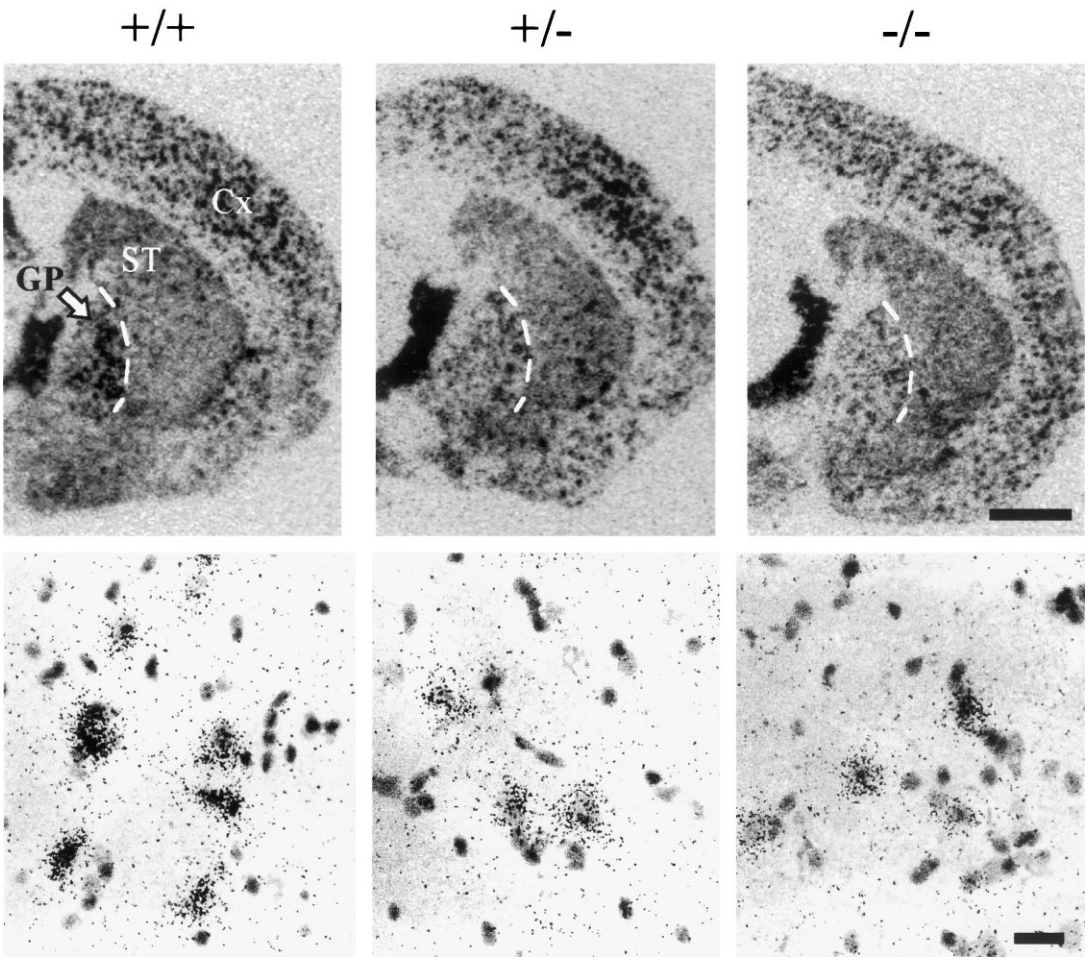

Fig. 2. Low-power magnification of film autoradiograms (upper row) and high-power microphotographs obtained from hybridized hematoxylin-counterstained sections (lower row), showing a reduced expression of GAD_{67} mRNA in the GP of D_2 dopamine receptor-deficient mice. Scale bars = 1 mm (upper row), 20 μm (lower row).

approximately 70% of the D_2 dopamine receptor +/+ mice values (Table 2, Fig. 2). A slighter reduction in pallidal GAD_{67} mRNA expression was also found in D_2 dopamine receptor +/− mice, although it did not reach statistical significance. The expression of GAD_{67} mRNA in cortical neurons (parietal cortex) was also measured on the same tissue sections, and no differences were found between wild-type and D_2 dopamine receptor-deficient mice (Table 2).

Subthalamic nucleus

The activity of COI in the STN, assayed on tissue sections by enzyme histochemistry, was significantly increased in D_2 dopamine receptor −/− and +/− mice, compared to wild-type mice (Table 3, Fig. 3). The study of COI mRNA expression at the cellular level by *in situ* hybridization also revealed a significantly increased expression in D_2 dopamine receptor −/− mice compared to controls, while D_2 dopamine receptor +/− mice showed a smaller, non-significant increase (Table 3, Fig. 3).

Basal ganglia output nuclei

In contrast to what has been repeatedly reported in animal models of PD, D_2 receptor-deficient mice did not show any changes in GAD_{67} mRNA expression in the substantia nigra pars reticulata and entopeduncular nucleus (Table 2, Fig. 4). COI histochemical staining was also similar in D_2 dopamine receptor +/+ and D_2 dopamine receptor-deficient mice in the substantia nigra pars reticulata (not shown).

DISCUSSION

D_2 dopamine receptor deficiency leads to parkinsonian-like changes in both striatopallidal and striatonigral neurons

Increased expression of enkephalin mRNA and decreased expression of substance P mRNA follow the destruction of mesencephalic dopaminergic neurons in different animal models of PD.[4,16,19,35] The absence of both functional copies of the D_2 receptor gene led to a striatal neurochemical phenotype that closely resembled that observed in animal Parkinsonism (Fig. 1). Similar findings for F_2 D_2 dopamine receptor −/− mice have been reported by others.[5] We further demonstrated that +/− mice also differ from wild-type mice on the basis of substance P and enkephalin striatal expression. Interestingly, the increase in enkephalin mRNA expression seemed to depend on gene dosage, whereas that of substance P did not (the degree of change was similar for +/− and −/− mice). Saturation binding of [^{3}H]nemonapride to mouse striatal membranes demonstrated a complete absence of specific binding in D_2 receptor −/− mice and a 50% reduction of

Table 3. Cytochrome oxidase histochemical staining and mRNA expression in the subthalamic nucleus of D_2 dopamine receptor-deficient mice

	+/+	+/−	−/−
Enzyme histochemistry (ROD)	16.67 ± 0.28	18.43 ± 0.55*	19.07 ± 0.77*
In situ hybridization (grains/μm^2)	0.70 ± 0.06	0.91 ± 0.07	1.00 ± 0.09†

Data are the mean ± S.E.M. of eight to 11 mice per group (enzyme histochemistry) or five to six mice per group (*in situ* hybridization). ROD, relative optical density. *$P < 0.05$ vs +/+, Dunn's method after significant Kruskal–Wallis one-way ANOVA on ranks ($H = 10.198$, 2 d.f., $P = 0.006$). †$P < 0.05$ vs +/+, Tukey's test after significant one-way ANOVA ($F_{2,24} = 16.2$, $P < 0.001$).

binding sites in +/− mice.[23] Thus, it seems that the appropriate control of the expression of these striatal peptides requires an intact population of striatal D_2 receptors.

The observed decrease in substance P mRNA expression could not be predicted on the basis of the classical basal ganglia model, which proposed that striatonigral neurons are under the control of D_1 receptors (see Introduction). These results suggest that the direct and indirect basal ganglia pathways are not exclusively under the control of one kind of dopamine receptor. In good agreement with our observations, recent reports stressed the existence of a high degree of co-localization of D_1 and D_2 receptors in striatofugal neurons,[2,38] as well as interactions between striatopallidal and striatonigral neurons mediated by axon collaterals and/or striatal interneurons.[3,7,17,47] However, changes in substance P mRNA expression in D_2 receptor-deficient mice could have followed modifications in extracellular dopamine clearance or D_1 receptor density, which have been shown to occur in D_2 receptor-deficient mice.[12,24] The latter possibility seems less likely, however, since significant changes in D_1 receptor density and dopamine clearance only occur in D_2 receptor −/− mice, whereas changes of substance P mRNA expression were of similar magnitude in +/− and −/− mice.

The decrease in striatal substance P mRNA content is likely not the result of a developmental compensatory mechanism. In order to balance the increased influence of the indirect pathway on the output nuclei, a compensatory hyperactivity of the inhibitory direct pathway would have occurred. Decreased expression of substance P mRNA is supposed to reflect a reduced activity of the GABAergic striatonigral pathway and determine output nuclei disinhibition (like in PD), and consequently it should have potentiated the behavioral consequences of D_2 receptor deficiency.

Lack of D_2 dopamine receptor stimulation leads to subthalamic nucleus hyperactivity

STN hyperactivity has been established as one of the cardinal pathophysiological hallmarks of Parkinsonism (see Introduction). It was originally proposed that, in Parkinsonism, STN hyperactivity follows the lack of endogenous dopamine acting on striatal D_2 receptors.[11,44] No compelling evidence has ever been provided, however, demonstrating that STN hyperactivity is the consequence of a lack of stimulation of D_2 dopamine receptors. Lack of striatal D_2 receptor stimulation was proposed to lead to hyperactivity of striatopallidal neurons and GP inhibition, resulting in STN disinhibition. Recent work raised concerns about this hypothesis. First, Hassani *et al.*[18] found that the STN remains hyperactive in 6-hydroxydopamine-lesioned rats after lesions of the GP,

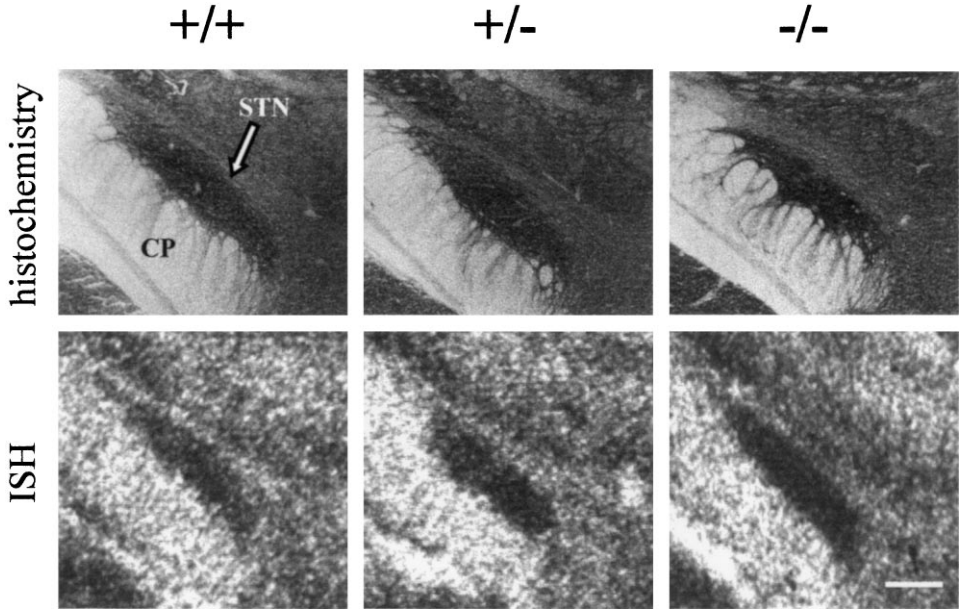

Fig. 3. Upper row: low-power microphotographs showing COI activity in the STN, as assessed by enzyme histochemistry. Lower row: low-power magnification of film autoradiograms obtained from sections hybridized with a ^{35}S-labeled probe directed against mouse COI mRNA. Note the increased activity and increased expression of COI in the STN of mice lacking D_2 dopamine receptors when compared with their congenic N_5 wild-type siblings. ISH, *in situ* hybridization. Scale bars = 0.5 mm.

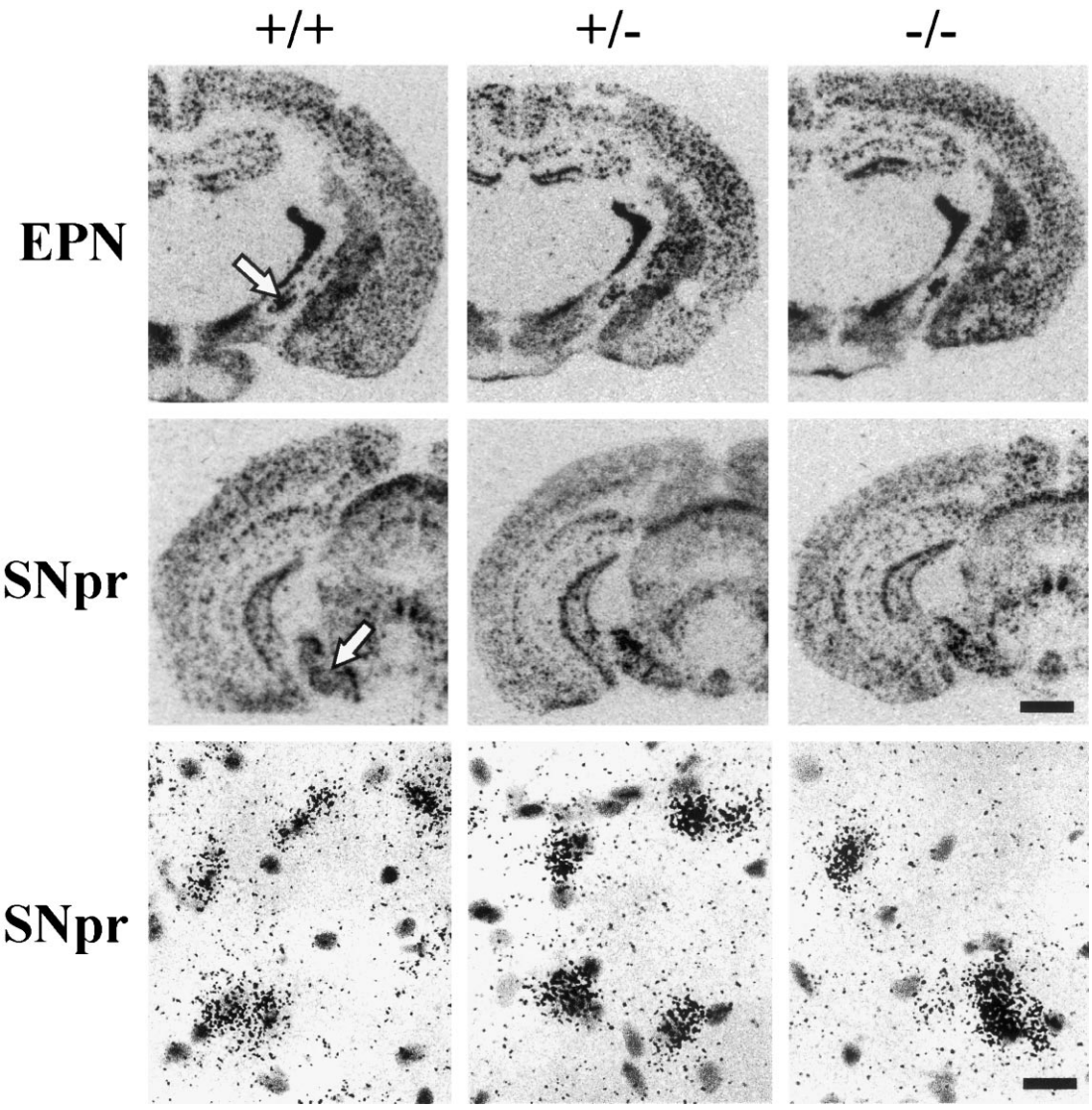

Fig. 4. Expression of GAD_{67} mRNA in the entopeduncular nucleus and substantia nigra pars reticulata of D_2 dopamine receptor-deficient mice and their N_5 wild-type siblings. Upper rows: low-power magnifications of film autoradiograms. Scale bar = 1 mm. Lower row: high-power microphotographs obtained from hybridized hematoxylin-counterstained mesencephalic sections. Scale bar = 20 μm. EPN, entopeduncular nucleus; SNpr, substantia nigra pars reticulata.

and that lesions of the GP lead to minimal hyperactivity of the STN in normal animals. Second, it was reported that the expression of GAD_{67} in the GP is normal or even higher in animal models of PD.[10,26] According to the model, GAD_{67} GP expression should be reduced, however. Thus, the mechanism leading to STN hyperactivity in Parkinsonism is not yet understood.

Our work provides strong evidence in support of impaired D_2 receptor function being enough to produce STN hyperactivity. Furthermore, it suggests that endogenous dopamine acting on D_2 receptors maintains the normal level of GAD_{67} expression in the GP. In addition, our findings (increased expression and activity of COI in the STN, and decreased GAD_{67} mRNA expression in the GP) are fully consistent with the classical view that hyperactivity of striatopallidal neurons leads to GP inhibition and STN disinhibition, although they should not be taken as a demonstration of this hypothesis. The lack of D_2 receptors in other brain regions, such as the cerebral cortex[8,43] or the STN itself,[21,30] could have contributed to STN hyperactivity in D_2 receptor-deficient mice as well.

That GAD_{67} mRNA expression was reduced in the GP of D_2 receptor-deficient mice was a surprising finding. Destruction of the mesostriatal dopaminergic pathway usually produces no change or leads to an increase in pallidal GAD_{67} mRNA expression.[10,26] It could be that pallidal GAD_{67} expression is not solely under the control of D_2 receptors, but that other dopamine receptors might influence it as well. Recent findings showing that D_1-type receptor-selective drugs modify the expression of Fos in the GP support the latter.[34] Furthermore, D_1-type receptor agonists are able to modify the firing rate of GP neurons.[9] The effects of D_1-type receptors on GP neurons could be mediated, at least partially, by axon collaterals from D_1 receptor-containing striatonigral neurons projecting into the GP.[22]

Relationship between behavioral and neurochemical phenotypes

Behaviorally, D_2 dopamine receptor −/− mice do not display a parkinsonian phenotype. No rigidity or tremor are observed in these mutant mice.[24] However, D_2 dopamine

receptor −/− mice exhibit a reduced number of movement initiations when tested in an open field. Thus, total horizontal activity of D_2 dopamine receptor −/− mice is reduced to half the total distance traveled by their N_5 congenic D_2 dopamine receptor +/+ siblings. In contrast with what occurs in PD, the initiated locomotor acts are performed at normal speed.

Whereas the behavioral phenotype of D_2 dopamine receptor −/− mice only partially resembles that of Parkinsonism, their basal ganglia neurochemical phenotype strikingly parallels that observed in animal models of PD, with the remarkable exception of GAD_{67} mRNA expression in the basal ganglia output nuclei. GAD_{67} mRNA expression is typically increased in the output nuclei of animals with nigrostriatal lesions,[20,37,39,42] but not in D_2 receptor-deficient mice. Increased activity in the basal ganglia output nuclei (reflected in increased GAD_{67} mRNA expression) is presumed to determine inhibition of thalamocortical circuits and the clinical signs of Parkinsonism.[11] It can be suggested that, in D_2 receptor-deficient mice, STN hyperactivity cannot by itself induce a change in the output nuclei strong enough to produce a complete parkinsonian behavioral phenotype. In contrast, the lack of endogenous stimulation of both D_1 and D_2 receptors might cooperate in determining output nuclei hyperactivity and behavioral changes in animals with nigrostriatal lesions.

It should be remembered that D_2 dopamine receptor-deficient mice develop in the absence of a functional receptor protein, while adult animals depleted of dopamine after a lesion of nigral cells with toxins and humans with PD have successfully completed their development and still express the D_2 receptor protein. Thus, alternative developmental programs could have compensated for the neurochemical changes occurring at the striatum and STN in D_2 dopamine receptor-deficient mice, determining an attenuated parkinsonian phenotype. It should be stressed, however, that D_2 dopamine receptor-deficient mice do not show gross anatomical or histological CNS abnormalities, resemble wild-type mice in most neurochemical and behavioral measures, and seem to have not developed compensatory mechanisms in dopaminergic neurotransmission.[12,23,24] Further work aimed at selectively blocking D_2 dopamine receptor function in fully developed animals will allow us to clarify the relevance of D_2 receptors in determining the behavioral parkinsonian phenotype.

Finally, it is important to consider the existence of other experimental approaches to undertake the study of basal ganglia function and the pathophysiology of PD. Recent reports stressed the existence of subtle changes in the firing pattern of neurons in the GP, STN and basal ganglia output nuclei in animal models of PD.[12,18,27,29] Studies aimed at characterizing the electrophysiological activity of the basal ganglia in D_2 receptor-deficient mice will probably help to clarify the role of the protein in the genesis of these changes, and the relationship between neurochemical and electrophysiological indices of neuronal activity.

CONCLUSION

The basal ganglia neurochemical phenotype of D_2 receptor-deficient mice resembles that of animals chronically depleted of dopamine after toxin-induced lesions. The most important finding of our study is that the lack of D_2 receptors in the mutant mice is sufficient to induce STN hyperactivity (a cardinal pathophysiological change in Parkinsonism), as demonstrated by STN increased COI histochemical staining and COI mRNA expression. The mechanisms leading to the "parkinsonian" neurochemical phenotype in these mice could be different, however, from those involved in the pathophysiology of PD. Thus, GAD_{67} mRNA expression is not modified or even increased in the GP of animals with nigrostriatal lesions, but it is reduced in D_2 receptor-deficient mice. Finally, we found that GAD_{67} mRNA expression in the basal ganglia output nuclei is not increased in D_2 dopamine receptor-deficient mice as it is in Parkinsonism. This difference could be related to the distinct behavioral phenotypes of D_2 dopamine receptor-deficient mice and parkinsonian animals.

Acknowledgements—This study was supported by INSERM and CNRS (France), Fundación Antorchas and CONICET (Argentina), the National Institute of Drug Abuse (USA), the National Parkinson Foundation (Miami, FL, USA), ANPCYT (Argentina), and ECOS (France).

REFERENCES

1. Accili D., Fishburn C. S., Drago J., Steiner H., Lachowicz J. E., Park B. -H., Gauda E. B., Lee E. J., Cool M. H., Sibley D. R., Gerfen C. R., Westphal H. and Fuchs S. (1996) A targeted mutation of the D3 dopamine receptor gene is associated with hyperactivity in mice. *Proc. natn. Acad. Sci. USA* **93,** 1945–1949.
2. Aizman O., Brismar H., Uhlen P., Zettergren E., Levey A. I., Forssberg H., Greengard P. and Aperia A. (2000) Anatomical and physiological evidence for D1 and D2 dopamine receptor colocalization in neostriatal neurons. *Nat. Neurosci.* **3,** 226–230.
3. Aronin N., Chase K. and DiFiglia M. (1986) Glutamic acid decarboxylase and enkephalin immunoreactive axon terminals in the rat neostriatum synapse with striatonigral neurons. *Brain Res.* **365,** 151–158.
4. Asselin M. C., Soghomonian J. J., Cote P. Y. and Parent A. (1994) Striatal changes in preproenkephalin mRNA levels in parkinsonian monkeys. *NeuroReport* **5,** 2137–2140.
5. Baik J. -H., Picetti R., Dalardi A., Thirlet G., Dierich A., Depaulis A., Le Meur M. and Borrelli E. (1995) Parkinsonian-like locomotor impairment in mice lacking dopamine D2 receptors. *Nature* **377,** 424–428.
6. Bisconte J. C., Fulcrand J. and Marty R. (1968) Analyse radioautographique dans le systeme nerveux centrale par photometrie et cartographie combinees. *C. r. Séanc. Soc. Biol.* **161,** 2178–2182.
7. Bolam J. P. and Izzo P. N. (1988) The postsynaptic targets of substance P immunoreactive terminals in the rat neostriatum with particular reference to identified spiny striatonigral neurons. *Expl Brain Res.* **70,** 361–377.
8. Bouthenet M. L., Martres M. P., Sales N. and Schwartz J. (1987) A detailed mapping of dopamine D-2 receptors in rat central nervous system by autoradiography with [^{125}I]iodosulpride. *Neuroscience* **20,** 117–155.
9. Carlson J. H., Bergstrom D. A., Demo S. D. and Walters J. R. (1990) Nigrostriatal lesion alters neurophysiological responses to selective and non-selective D1 and D2 dopamine agonists in rat globus pallidus. *Synapse* **5,** 83–93.
10. Chesselet M.-F. and Delfs J. M. (1996) Basal ganglia and movement disorders: an update. *Trends Neurosci.* **19,** 417–422.
11. DeLong M. R. (1990) Primate models of movement disorders of basal ganglia origin. *Trends Neurosci.* **13,** 281–285.
12. Dickinson S. D., Sabeti J., Larson G. A., Giardina K., Rubinstein M., Kelly M. A., Grandy D. K., Low M. J., Gerhardt G. A. and Zahniser N. R. (1999)

Dopamine D2 receptor-deficient mice exhibit decreased dopamine transporter function but no changes in dopamine release in dorsal striatum. *J. Neurochem.* **72,** 148–156.
13. Drago J., Gerfen C. R., Lachowicz J. E., Steiner H., Hollon T. R., Love P. E., Ooi G. T., Grinberg A., Lee E. J., Huang S. P., Bartlett P. F., Jose P. A., Sibley D. R. and Westphal H. (1994) Altered striatal function in a mutant mouse lacking D1A dopamine receptors. *Proc. natn. Acad. Sci. USA* **91,** 12,564–12,568.
14. Filion M. and Tremblay L. (1991) Abnormal spontaneous activity of globus pallidus neurons in monkey with MPTP-induced Parkinsonism. *Brain Res.* **547,** 142–151.
15. Fontaine B., Sassoon D., Buckingham M. and Changeux J. P. (1988) Detection of the nicotinic acetylcholine receptor subunit mRNA by *in situ* hybridization at the neuromuscular junction of 15-day-old chick striatal muscles. *Eur. molec. Biol. Org. J.* **7,** 603–609.
16. Gerfen C. R., Engber T. M., Mathan L. C., Susel Z., Chase T. N., Monsma F. J. and Sibley D. R. (1990) D1 and D2 dopamine receptor regulated gene expression of striatonigral and striatopallidal neurons. *Science* **250,** 1429–1432.
17. Gerfen C. R., Keefe K. A. and Gauda E. B. (1995) D1 and D2 dopamine receptor function in the striatum: coactivation of D1 and D2 dopamine receptors on separate populations of neurons results in potentiated immediate early gene responses in D1-containing neurons. *J. Neurosci.* **15,** 8167–8176.
18. Hassani O.-K., Mouroux M. and Féger J. (1996) Increased subthalamic neuronal activity after nigral dopaminergic lesion independent of disinhibition via the globus pallidus. *Neuroscience* **72,** 105–115.
19. Herrero M.-T., Augood S. J., Hirsch E. C., Javoy-Agid F., Luquin M. R., Agid Y., Obeso J. A. and Emson P. C. (1995) Effects of L-DOPA on preproenkephalin and preprotachykinin gene expression in the MPTP-treated monkey striatum. *Neuroscience* **68,** 1189–1198.
20. Herrero M.-T., Levy R., Ruberg M., Javoy-Agid F., Luquin M. R., Agid Y., Hirsch E. C. and Obeso J. A. (1996) Glutamic acid decarboxylase mRNA expression in medial and lateral pallidal neurons in MPTP-treated monkeys and patients with Parkinson's disease. *Adv. Neurol.* **69,** 209–216.
21. Johnson A. E., Coirini H., Kallstrom L. and Wiesel F. (1994) Characterization of dopamine receptor binding sites in the subthalamic nucleus. *NeuroReport* **5,** 1836–1838.
22. Kawaguchi Y., Wilson C. J. and Emson P. C. (1990) Projection subtypes of neostriatal matrix cells revealed by intracellular injection of biocytin. *J. Neurosci.* **10,** 3421–3438.
23. Kelly M. A., Rubinstein M., Asa S. L., Zhang G., Saez C., Bunzow J. R., Allen R. G., Hnasko R., Ben-Jonathan N., Grandy D. K. and Low M. J. (1997) Pituitary lactotroph hyperplasia and chronic hyperprolactinemia in dopamine D2 receptor-deficient mice. *Neuron* **19,** 103–113.
24. Kelly M. A., Rubinstein M., Phillips T. J., Lessov C. N., Burkhart-Kasch S., Zhang G., Bunzow J. R., Fang Y., Gerhardt G. A., Grandy D. K. and Low M. J. (1998) Locomotor activity in D2 dopamine receptor-deficient mice is determined by gene dosage, genetic background, and developmental adaptations. *J. Neurosci.* **18,** 3470–3479.
25. Levy R., Herrero M.-T., Ruberg M., Villares J., Faucheux B., Guridi J., Luquin M. R., Javoy-Agid F., Obeso J. A., Agid Y. and Hirsch E. C. (1995) Effects of nigrostriatal denervation and L-dopa therapy on the GABAergic neurons of the striatum in MPTP-treated monkeys and Parkinson's disease: an *in situ* hybridization study of GAD_{67} mRNA. *Eur. J. Neurosci.* **7,** 1199–1209.
26. Levy R., Hazrati L.-N., Herrero M.-T., Vila M., Hassani O.-K., Mouroux M., Ruberg M., Asensi H., Agid Y., Féger J., Obeso J. A., Parent A. and Hirsch E. C. (1997) Re-evaluation of the functional anatomy of the basal ganglia in normal and parkinsonian states. *Neuroscience* **76,** 335–343.
27. Miller W. C. and DeLong M. R. (1988) Parkinsonian symptomatology: an anatomical and physiological analysis. *Ann. N. Y. Acad. Sci.* **515,** 287–302.
28. Mitchell I. J., Clark C. E., Boyce S., Robertson R. G., Peggs D., Sambrook M. A. and Crossman A. R. (1989) Neural mechanisms underlying parkinsonian symptoms based upon regional uptake of 2-desoxyglucose in monkeys exposed to MPTP. *Neuroscience* **32,** 213–226.
29. Murer M. G., Riquelme L. A., Tseng K. and Pazo J. H. (1997) Substantia nigra pars reticulata single unit activity in normal and 6-OHDA-lesioned rats: effects of intrastriatal apomorphine and subthalamic lesions. *Synapse* **27,** 278–293.
30. Murer M. G., Ferrario J., Delfino M., Dziewczapolski G., Gershanik O. S. and Raisman-Vozari R. (1999) Increased [^{125}I]sulpiride binding in the subthalamic nucleus of rats with nigrostriatal lesions. *NeuroReport* **10,** 1501–1505.
31. Pan H. S. and Walters J. R. (1988) Unilateral lesion of the nigrostriatal pathway decreases the firing rate and alters the firing pattern of globus pallidus neurons in the rat. *Synapse* **2,** 650–656.
32. Porter R. H. P., Greene J. G., Higgins D. S. and Greenamyre J. T. (1994) Polysynaptic regulation of glutamate receptors and mitochondrial enzyme activities in the basal ganglia of rats with unilateral dopamine depletion. *J. Neurosci.* **14,** 7192–7199.
33. Rubinstein M., Phillips T. J., Bunzow J. R., Falzone T. L., Dziewczapolski G., Zhang G., Fang Y., Larson J. L., McDougall J. A., Chester J. A., Saez C., Pugsley T. A., Gershanik O., Low M. J. and Grandy D. K. (1997) Mice lacking dopamine D4 receptors are supersensitive to ethanol, cocaine, and methamphetamine. *Cell* **90,** 991–1001.
34. Ruskin D. N. and Marshall J. F. (1995) D1 dopamine receptors influence Fos immunoreactivity in the globus pallidus and subthalamic nucleus of intact and nigrostriatal-lesioned rats. *Brain Res.* **703,** 156–164.
35. Salin P., Dziewczapolski G., Gershanik O., Nieoullon A. and Raisman-Vozari R. (1997) Differential regional effects of long-term L-DOPA treatment on preproenkephalin and preprotachykinin gene expression in the striatum of 6-hydroxydopamine-lesioned rats. *Molec. Brain Res.* **47,** 311–321.
36. Smith Y., Bevan M. D., Shink E. and Bolam J. P. (1998) Microcircuity of the direct and indirect pathways of the basal ganglia. *Neuroscience* **86,** 353–387.
37. Soghomonian J. J., Pedneault S., Audet G. and Parent A. (1994) Increased glutamate decarboxylase mRNA levels in the striatum and pallidum of MPTP-treated primates. *J. Neurosci.* **14,** 6256–6265.
38. Surmeier D. J., Song W. J. and Yan Z. (1996) Coordinated expression of dopamine receptors in neostriatal medium spiny neurons. *J. Neurosci.* **16,** 6579–6591.
39. Vila M., Herrero M.-T., Levy R., Faucheux B., Ruberg M., Guillen J., Luquin M. R., Guridi J., Javoy-Agid F., Agid Y., Obeso J. A. and Hirsch E. C. (1996) Consequences of nigrostriatal denervation on the GABAergic neurons of substantia nigra pars reticulata and superior colliculus in parkinsonian syndromes. *Neurology* **46,** 802–809.
40. Vila M., Levy R., Herrero M.-T., Faucheux B., Obeso J. A., Agid Y. and Hirsch E. C. (1996) Metabolic activity of the basal ganglia in parkinsonian syndromes in human and non-human primates: a cytochrome oxidase histochemistry study. *Neuroscience* **71,** 903–912.
41. Vila M., Levy R., Herrero M.-T., Ruberg M., Faucheux B., Obeso J. A., Agid Y. and Hirsch E. C. (1997) Consequences of nigrostriatal denervation on the functioning of the basal ganglia in human and non-human primates: an *in situ* hybridization study of cytochrome oxidase subunit I mRNA. *J. Neurosci.* **17,** 765–773.
42. Vila M., Marin C., Ruberg M., Jimenez A., Raisman-Vozari R., Agid Y., Tolosa E. and Hirsch E. C. (1999) Systemic administration of NMDA and AMPA receptor antagonists reverses the neurochemical changes induced by nigrostriatal denervation in the basal ganglia. *J. Neurochem.* **73,** 344–352.
43. Weiner D. M., Levey A. I., Sunahara R. K., Niznik H. B., O'Dowd B. F., Seeman P. and Brann M. (1991) D1 and D2 dopamine receptor mRNA in rat brain. *Proc. natn. Acad. Sci. USA* **88,** 1859–1863.
44. Wichmann T. and DeLong M. R. (1996) Functional and pathophysiological models of the basal ganglia. *Curr. Opin. Neurobiol.* **6,** 751–758.
45. Wong-Riley M. T. T. (1979) Changes in the visual system of monocularly sutured or enucleated cats demonstrable with cytochrome oxidase histochemistry. *Brain Res.* **171,** 11–28.
46. Xu M., Moratalla R., Gold L. H., Hiroi N., Graybiel A. M. and Tonegawa S. (1994) Dopamine D1 receptor mutant mice are deficient in striatal expression of dynorphin and in dopamine-mediated behavioral responses. *Cell* **79,** 729–742.
47. Yung K. K. L., Smith A. D., Levey A. I. and Bolam J. P. (1996) Synaptic connections between spiny neurons of the direct and indirect pathways in the neostriatum of the rat: evidence from dopamine receptor and neuropeptide immunostaining. *Eur. J. Neurosci.* **8,** 861–869.

(*Accepted* 3 *May* 2000)