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High-frequency stimulation of the subthalamic nucleus prolongs the increase in striatal dopamine induced by acute L-DOPA in dopaminergic denervated rats

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Abstract

High-frequency stimulation of the subthalamic nucleus (STN-HFS) is a powerful approach for treating the motor symptoms of Parkinson's disease. It results in clinical improvement in PD patients, further reducing L-3, 4-dihydroxyphenylalanine (L-DOPA) requirement and thus L-DOPA-induced dyskinesia. However, it remains unclear how STN-HFS modifies the response to L-DOPA. We investigated the effect of STN-HFS on striatal extracellular concentrations of dopamine and its metabolites following acute L-DOPA administration in intact or partially dopaminergic denervated (DA PL) rats. L-DOPA treatment significantly increased striatal dopamine levels in intact and DA PL animals, with the maximal effect observed 1 h after L-DOPA injection. This increase was more pronounced in DA PL rats (ipsilateral to the lesion) than in intact animals. It remained fairly stable 1 h after the maximal effect of L-DOPA and then decreased towards basal values. STN-HFS in intact rats had no effect on the maximal L-DOPA-induced increase in striatal extracellular dopamine concentration or the return to basal values, the profiles observed being similar to those for non-stimulated intact animals. Conversely, STN-HFS amplified the L-DOPA-induced increase in striatal dopamine levels during the stimulation period (1 h) in DA PL rats, and this increase was sustained throughout the post-stimulation period (2.5 h), without the return to basal levels observed in stimulated intact and non-stimulated rats. These new neurochemical data suggest that STN-HFS interferes with L-DOPA effects, probably synergically, by stabilising dopamine levels in the striatum, and shed light on the mechanisms of STN-HFS in PD.

Introduction

L-3, 4-dihydroxyphenylalanine (L-DOPA) remains the most effective treatment for Parkinson's disease (PD). However long-term L-DOPA therapy induces many motor fluctuations (Fahn, 1974; Marsden and Parkes, 1976). L-DOPA induces a short-duration response (SDR) and a long-duration response (LDR) in patients with PD (Muenter and Tyce, 1971; Nutt *et al*., 1992; 1995). The SDR is a clinical improvement that lasts several hours after a single dose of L-DOPA whereas the LDR is a sustained improvement in parkinsonian following chronic L-DOPA therapy, persisting for several days after the end of treatment. Many PD patients experience changes in the therapeutic response to L-DOPA and dopamine agonists, such as apomorphine (Marsden and Parkes, 1976; Marsden, 1982; Nutt *et al*., 1992; 1995). The duration of the SDR and LDR is proportional to disease severity in patients with mild, moderate and severe disease (Muenter and Tyce, 1971; Contin *et al*., 1990; Nutt *et al*., 1992; Quattrone and Zappia, 1993; Zappia *et al.*, 1997; 1999; Parkinson Study Group, 2004). Fluctuations in motor responses, such as "wearing-off" and "on-off" fluctuations, as well as L-DOPA-induced dyskinesia, are commonly observed in patients in advanced stages of PD. As the LDR is thought to be due to the slow release of L-DOPA from residual presynaptic neurones (Quattrone and Zappia, 1993; Nutt and Holford, 1996), this response becomes less important with respect to SDR in advanced PD. It has therefore been suggested that the pulsatile administration of L-DOPA is the principal cause of motor fluctuations (Juncos *et al*., 1989), because of the induction of abnormal plasticity in the basal ganglia, leading to the induction of dyskinesia and a decrease in the "on" duration (Mouradian *et al*., 1990). For this reason, continuous dopaminergic stimulation occupies a particularly important position in therapeutic strategies (Obeso *et al*., 2000; Olanow and Obeso, 2000; Olanow *et al*., 2000; 2001).

High-frequency stimulation (HFS) of the subthalamic nucleus (STN) has been reported to improve all motor symptoms in PD patients, particularly in those who experience motor fluctuations, such as the wearing-off phenomenon (Limousin *et al*., 1998; Moro *et al.*, 1999; Fraix *et al.*, 2000; Krack *et al*., 2003), but the mechanisms underlying the improvement in symptoms remain unclear (Benabid *et al*.,

2002; Dostrovsky and Lozano, 2002; McIntyre *et al*., 2004). It also remains unclear whether STN-HFS interferes with L-DOPA-induced changes in striatal dopamine content, by attenuating fluctuations in particular. De la Fuente-Fernandez *et al*. (2001) have shown that fluctuations in synaptic dopamine concentrations in the striatum precede the clinically apparent wearing-off phenomenon. They suggested that an increase in dopamine turnover might be involved in L-DOPA-related motor complications.

In this study, we compared the effects of acute L-DOPA treatment with and without STN-HFS (according to the procedures used for treating PD in humans) on the striatal extracellular content of dopamine (DA) and its metabolites, in normal and partially dopaminergic-lesioned rats, by means of *in vivo* intracerebral microdialysis. Our data provide insight into the interactions between the effects of L-DOPA treatment and STN-HFS on dopaminergic neurotransmission.

Materials and Methods

Animals

We used 55 adult male Sprague-Dawley rats (Janvier, Le Genest-St-Isle, France) weighing 200 to 380g, housed under standard laboratory conditions (12 h dark/light cycle), with food and water provided *ad libitum*. Protocols conformed to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (publication 865-23) and French Ministry of Agriculture regulations (authorisation number 38-R 1001).

Lesion procedure

For partial SNc lesioning, all animals were anaesthetised with chloral hydrate (400 mg/kg i.p.) and secured in a Kopf stereotaxic apparatus (Phymep, Paris, France). We treated 25 animals with desipramine (25 mg/kg, s.c.), to protect noradrenergic neurons, and then injected 3 μg of 6-hydroxydopamine (6-OHDA)(Sigma, St Quentin-Fallavier, France) dissolved in 1 μl of sterile 0.9 % NaCl and 0.2% ascorbic acid into the left SNc of these animals, at a flow rate of 0.5 μl/min. The stereotaxic co-ordinates of the injection site were anteroposterior (AP), + 3 mm, lateral (L), + 2.4 mm, and dorsoventral (DV), -7.1 mm, with the incisor bar at +3.3 mm below the interaural plane. All stereotaxic co-ordinates cited here are according to the stereotaxic atlas of Paxinos and Watson (1982). Animals were kept warm after the injections and were allowed to recover from anaesthesia. They were returned to the animal facility for three weeks, to allow the degeneration of dopaminergic neurons induced by the neurotoxin to stabilise, and were then processed for microdialysis experiments.

Implantation of microdialysis probes and stimulating electrode

Normal (*n*=30) and partially 6-OHDA SNc lesioned (PL) (*n*=25) rats were anaesthetised by inhalation (1 l/min) of a 5% halothane/air (22% O_2, 78% N_2) mixture and mounted in a stereotaxic frame (David Kopf Instruments, Tajunga, CA). Anaesthesia was maintained with an inhaled 1% halothane/air mixture

(1 l/min). The dorsal skull was exposed, and holes were drilled to facilitate the bilateral implantation of the dialysis probes into the striatum, and the unilateral (left side, i.e. ipsilateral to the 6-OHDA injection) implantation of the stimulation electrode in the STN. The stereotaxic co-ordinates used were (relative to bregma): (1) microdialysis probe (into striatum): AP, + 1 mm; L, +/- 3 mm; V, - 7 mm; (2) stimulation electrode (into STN): AP, -3.7 mm; L, 2.4 mm; V, -7.8 mm. During implantation and microdialysis experiments, body temperature was maintained at 37°C with a feedback-controlled heating pad (Harvard Apparatus, Edenbridge, UK).

Electrical stimulation of the STN

Concentric stimulating bipolar electrodes (outer diameter 250 µm, SNEX 100, Rhodes Medical Instruments, Woodland Hills, CA) were used. Stimuli were delivered over a one-hour period, with a World Precision Instrument (Stevenage, UK) acupulser and a stimulus isolation unit. Stimulation parameters corresponded to those used in clinical practice (frequency 130 Hz, 60 µs rectangular pulse width and 200 µA intensity). At the end of each experiment, an electrical lesion was created in the STN so that the position of the electrode could be checked post-mortem.

Microdialysis

Home-made microdialysis probes were prepared and used as previously described (Windels *et al.*, 2000; Bruet *et al*., 2003). They consisted of a concentric arrangement of a stainless steel tube (outer diameter, 0.4 mm, (Phymep, Paris, France), and polyethylene tubing (outer diameter, 1.09 mm; inner diameter, 0.38 mm) (Phymep) into which we inserted a piece of silica tubing (outer diameter, 150 µm; inner diameter, 75 µm) (Phymep). The silica tubing extended beyond the distal end of the steel tube and was covered with a cuprophan tubular dialysis membrane (Hospal, Lyons, France), sealed at the bottom with epoxy glue. The length of the dialysis membrane was adapted for the rat brain nucleus studied (3 mm for the striatum).

The perfusion liquid flowed out of the distal end of the steel tube, passing proximally between the tube and the membrane (Tossman and Ungerstedt, 1986). The probes were perfused with artificial CSF (149 mM NaCl, 2.8 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$ and 5.4 mM glucose, pH=7.3) at a flow rate of 1 μL/min. Before implantation, each probe was tested *in vitro* in a standard catecholamine solution, to determine DA, DOPAC and HVA recovery (Tossman and Ungerstedt, 1986).

For microdialysis experiments, two experimental animal groups were used: non-stimulated (intact, n= 15; DA PL, n= 13) and stimulated (intact+STN-HFS, n= 15; DA PL+STN-HFS, n= 12). The dialysis probes were implanted in each animal and dialysates were collected at 15-minute intervals, for seven hours. The first eight fractions (2 h) were discarded to prevent effects due to parenchymal disturbance and to ensure that a steady-state was reached. The next four fractions (1 h) were collected for basal value determination. All animals then received an intraperitoneal injection of benserazide (12.5 mg/kg, Sigma, St Quentin-Fallavier, France) + L-DOPA (50 mg/kg, Sigma).

The non-stimulated rats were first used to evaluate the duration of the L-DOPA effect on extracellular DA content, in intact control and DA PL rats. The first eight fractions were discarded to prevent effects due to parenchymal disturbance and to ensure that a steady-state values were obtained. The next four fractions (1 h) were collected for basal value determination, and sixteen consecutive fractions (4 h) were then collected after L-DOPA injection: 6 fractions were used to determine the maximal effect of L-DOPA, and the remaining 10 fractions were collected to estimate the effect of L-DOPA over a period of 2.5 h.

We then carried out a similar experiment in stimulated rats. As for the non-stimulated animals, the first eight fractions were discarded to prevent effects due to parenchymal disturbance and to ensure that the values obtained corresponded to an approximate steady state. Four fractions (1 h) were then collected for basal value determination, followed by six fractions (1.5 hours) collected after L-DOPA injection to assess the maximal effect of this drug on striatal DA content. The next four fractions were collected

during STN-HFS for 1 h. A further six fractions were then collected to estimate post-stimulation effects over a period of 1.5 hours. All dialysates were collected automatically with a refrigerated autosampler (Univentor, Zejton, Malta) and stored at -80°C until analysis.

DA, DOPAC and HVA assays

DA, DOPAC and HVA concentrations in the dialysates were determined by high-performance liquid chromatography (HPLC), with electrochemical detection. The system consisted of a pump (Shimadzu model LC-10 AD, Shimadzu Europe, Munich, Germany), a refrigerated automatic injector (Famos model, Dionex, France), a reverse-phase Hypersil RP 18 analytical column (Aquasil 150x1 mm, particle size 3 µm; ThermoHypersil, les Ulis, France) and an electrochemical detector (Decade, Antec, The Netherlands) equipped with an analytical cell (type VT-03, Antec). Chromatograms were collected and treated with integration software (CLAS VP, Shimadzu, France).

The mobile phase consisted of sodium dihydrogen phosphate buffer (NaH_2PO_4, 50 mM; Merck), octane sulfonic-1 acid sodium salt (1.7 mM; Merck), disodium ethylenediamine tetra-acetic acid (Na_2-EDTA, 200 µM; Merck). The pH was adjusted to 3 with concentrated phosphoric acid (H_3PO_4), and 5% acetonitrile (ACN, Sigma) was added to the final solution. All solvents were filtered through Millipore filters with 0.22 µm pores (Millipore, France) before use.

The mobile phase was delivered by a pump with a flow rate of 60 µl/min. The working electrode potential was +750 mV, which represented the best compromise between the optimum oxidation potentials of DA, DOPAC and HVA. Detector sensitivity was 1 nA for DA, 50 nA for DOPAC and 10 nA for HVA. The running time for each determination was 15 minutes.

Histology

At the end of the microdialysis experiments, unlesioned rats (n=30) were killed by decapitation under deep anaesthesia. Their brains were quickly removed from the skull and frozen in isopentane at -30°C.

Lesioned animals (n=25) were perfused transcardially, under halothane anaesthesia, with 200 mL of 0.9 % NaCl, pH 7.2, followed by 300 mL of 4% paraformaldehyde in 0.1 M PBS, pH 7.4 (2.6 mM KCl, 1.4 mM KH_2PO_4, 136 mM NaCl, and 83 mM NaH_2PO_4). Brains were quickly removed and immersed overnight in 20% sucrose in 0.1 M phosphate buffer, pH 7.4, frozen in cooled (-40°C) isopentane, and stored at -30°C. Serial frontal sections (20 μm) were cut on a cryostat (Microm HM 500; Microm, Francheville, France). The correct locations of the microdialysis probes and stimulation electrode were checked by collecting several striatal and subthalamic tissue sections (Paxinos and Watson, 1982) and by counterstaining with cresyl violet. These histological controls were systematically carried out for all the animals in each experimental group. All animals presenting internal bleeding around the microdialysis probes or electrodes were excluded to prevent microdialysate contamination (*n*=4), as were animals with incorrectly positioned stimulation electrodes (n=8). We assessed the dopaminergic denervation induced by nigral 6-OHDA injection, by tyrosine hydroxylase (TH) immunostaining of striatal and nigral sections from the fixed brains of lesioned animals. Free-floating sections were thoroughly washed with TBS and incubated for 1 hour in 0.3% Triton X-100 in TBS (TBST) and 3 % normal goat serum (Sigma-Aldrich, St Quentin Fallavier, France). They were then incubated with primary antisera diluted in TBST containing 1 % normal goat serum for 24 h, with shaking, at 4°C. The antiserum was diluted 1:500 for TH staining (mouse monoclonal antibody; Chemicon, Temecula, CA). Antibody binding was detected by incubation with avidin-biotin-peroxidase conjugate (Vectastain ABC Elite, Vector Laboratories, Burlingame, CA), using 3,3'-diaminobenzidine as the chromagen. This substrate was applied to the sections for 2 to 5 min, as previously described (Guesdon *et al*., 1979). Sections were dehydrated through a graded series of ethanol solutions, cleared in xylene, mounted in DPX (DBH Laboratories Supplies, Poole, UK) and covered with a coverslip for microscopy.

The area of the SNc displaying cell loss and the area of the striatum displaying dopaminergic terminal loss were estimated by comparison with the unlesioned side or control values. These areas were determined by anatomical densitometry of TH immunolabelling, using a camera, and the mean optical

density was calculated with Autoradio V4.03 software (SAMBA Technologies, Meylan, France). These data were then compared with data previously obtained in similar conditions (Blanchard *et al*., 1995; Chritin *et al*., 1996; Bruet *et al*., 2001).

Data analysis

The basal levels of the measured substances are expressed as concentrations in dialysates. Basal DA, DOPAC and HVA levels in the striatum were analysed, for the various experimental groups, with a Mann-Whitney *U* test. For data expressed as a percentage of the basal value, the mean concentration of the four samples preceding L-DOPA injection was set at 100%. The effects of L-DOPA treatment and of STN-HFS on extracellular DA, DOPAC and HVA levels were analysed by one-way repeated measures ANOVA over time (Windels *et al.,* 2005; Boulet *et al.,* 2006). Dunnett's or Games-Howell tests were used for comparisons with prestimulation and/or baseline levels for L-DOPA effects. Values of $p<0.05$ were considered statistically significant.

Results

Control of the extent of the DA lesion and of the location of the electrode and microdialysis probes

One month after the unilateral injection of 6-OHDA, DA PL animals (n=25) showed weaker than normal TH immunolabelling in the lateral part of the ipsilateral SNc. In most DA PL rats, the total number of SNc TH+ neurons was about 50% lower than that on the unlesioned side (Fig. 1*A*). In the striatum of the same rats, DA nerve terminals, detected by TH immunolabelling, were preferentially lost from the dorsolateral (denervated) part of the striatum (Fig. 1*B*). In this area, TH immunolabelling (detected by optical density measurements) was a mean of 70% ($p<0.001$) lower than normal, whereas it was only 10 to 25% ($p<0.01$) lower in the medial region of the striatum (non denervated part). About 40% of the total striatal surface showed a loss of TH immunostaining.

However, some DA PL animals displayed severe (>80%, n=2) or moderate (<30%, n=3) lesions of the SNc and were discarded to ensure that the lesioned rat group was homogeneous.

Dense TH immunolabelling was detected throughout the SNc, the ventral tegmental area, the striatum, the nucleus accumbens, and the olfactory tubercles, on the intact side, in 6-OHDA-injected rats (Fig. 1*A*, *B*). Figures 1*D* and *F* illustrate the correct implantation of the microdialysis probe in the parenchyma of the striatum (Fig. 1*D*) and of the stimulation electrode in the STN (Fig. 1*F*). In DA PL animals, the microdialysis probe was located between the denervated and non-denervated parts of the striatum. Figure 1*F* shows the small electrical lesion (asterisk) created at the end of the experiment, indicating the point stimulated.

Basal extracellular DA, DOPAC and HVA levels in the striatum are increased by acute L-DOPA injection in intact and DA PL rats

The concentrations of DA, DOPAC and HVA were determined in the striatum of intact control (n=25) and DA PL (n=13) rats from the two experimental groups. Mean concentrations were 0.011 ± 0.0001 µM for DA, 1.7 ± 0.08 µM for DOPAC and 1.697 ± 0.085 µM for HVA in intact animals, and 0.0124 ±

0.0018 µM for DA, 1.137 ± 0.097 µM for DOPAC and 1.145 ± 0.085 µM for HVA in DA PL rats (Table 1). In DA PL rats, the concentration of striatal DA ipsilateral to the lesion tended to increase, as shown by comparisons with the unlesioned side and control values (0.0124 ± 0.0018 versus 0.011 ± 0.0019 µM). Partial DA denervation significantly decreased the levels of DOPAC (-33%, $p<0.001$) and of HVA (-32.5%, $p<0.001$), as shown by comparison with control values.

L-DOPA treatment significantly increased DA, DOPAC and HVA levels in the striatum of intact control and DA PL rats. These increases were more pronounced in DA PL animals (ipsilateral to the lesion) than in intact control rats and the mean variation for the two experimental groups was: +279% ($p<0.001$) versus +92% ($p<0.05$) for DA; +236% ($p<0.001$) versus +114% ($p<0.001$) for DOPAC, and +129% ($p<0.001$) versus +70% ($p<0.001$) for HVA (Table 1).

The DA turnover index was calculated as the concentration of DA metabolites (DOPAC+HVA) divided by the concentration of extracellular DA. It reflects the relationship between DA metabolism and DA release. This DA turnover index was not significantly affected by L-DOPA injection in either intact or DA PL rats, but it did increase over the 1 h and 2.5 h post-injection periods studied (Table 2).

Figure 2*A* illustrates the variations of striatal DA, DOPAC and HVA levels in intact (n=12) and DA PL (n=5) rats after acute L-DOPA injection only. In these non-stimulated animals, L-DOPA injection triggered a gradual increase in extracellular striatal DA levels during the period of 1.5 hours following the injection (data not shown). This increase was maximal in the last two of the six fractions collected during this period and the mean variation observed in these two fractions was used as a reference for L-DOPA effect. In intact rats, striatal DA levels remained high during the first hour of the post-L-DOPA effect period, decreasing towards basal values 2.5 h after the maximal effect of L-DOPA (Fig. 2*A*). The increase in striatal DA levels induced by L-DOPA injection was more pronounced in DA PL rats than in intact rats (+279% versus +92%, $p<0.001$) and remained fairly stable during the first hour of the post-L-DOPA effect period. As in intact animals, DA levels returned towards basal values 2.5 h after the

maximal effect of L-DOPA (Fig. 2*A*), but values remained significantly above these basal levels (Fig. 2*A*).

The L-DOPA-induced increase in striatal DOPAC and HVA levels followed a pattern similar to that for DA content, in both intact and DA PL rats, except that no pronounced return to basal levels was observed (Fig. 2*A*). In contrast, HVA levels remained high or increased still further during the microdialysis experiment.

Effect of STN-HFS on L-DOPA-induced increases of extracellular DA, DOPAC and HVA levels in the striatum in intact control and DA PL rats

The mean L-DOPA-induced increases in striatal DA, DOPAC and HVA levels under STN-HFS were 0.0191 ± 0.0017 µM, 4.182 ± 0.19 µM and 3.292 ±0.165 µM, respectively, for intact animals, and 0.0587 ± 0.0086 µM, 5.732 ± 0.488 µM and 3.928 ± 0.237 µM, respectively, for DA PL rats (Table 1).

In intact stimulated animals (n=13), STN-HFS did not significantly affect the L-DOPA-induced increase in striatal extracellular concentrations of DA and DOPAC, whereas it increased those of HVA (+24%, $p<0.001$), during the one-hour stimulation period (Table 1, Fig. 2*B*). During the post-stimulation period, DA levels significantly decreased (-52%, $p<0.05$ with respect to L-DOPA-induced levels) whereas HVA levels increased (+52%, $p<0.05$ with respect to L-DOPA-induced levels). DOPAC levels were not significantly affected. However, DA, DOPAC and HVA levels remained above basal levels 2.5 h after the maximal effect of L-DOPA in stimulated intact animals. The profile of change in levels of DA and its metabolites was similar to that in intact animals treated with L-DOPA, without STN-HFS. However, DA turnover index increased in intact animals during the stimulation (+63%, $p<0.05$ vs basal values) and post-stimulation (+117% , $p<0.001$ vs basal levels or +85%, $p<0.001$ vs L-DOPA effect) periods (Table 2).

In stimulated DA PL animals (n=8), STN-HFS significantly amplified the L-DOPA-induced increases in striatal extracellular DA, DOPAC and HVA levels. These levels increased by +94% (non-significant), +

168% ($p<0.05$) and + 114% ($p<0.05$) for DA, DOPAC and HVA, respectively, during the one-hour stimulation period (Table 1, Fig. 2*B*). In contrast to what was observed in non-stimulated DA PL (Fig 2*A*) and stimulated intact animals (Fig 2*B*), striatal extracellular DA, DOPAC and HVA levels remained high throughout the post-stimulation period in stimulated DA PL rats, strongly suggesting an effect of STN-HFS (Fig. 2*B*). Indeed, a comparison of DA levels in stimulated DA PL rats during the post-stimulation period with those obtained in similar non-stimulated animals at the same time points after L-DOPA treatment shown that DA levels tended to return to basal values in the absence of STN-HFS (Fig. 2*A*). In contrast to what was observed in intact rats, DA turnover index during the stimulation and post-stimulation periods was not significantly affected in DA PL animals (Table 2).

Discussion

The mechanisms by which STN-HFS improves motor symptoms in PD patients, particularly in those with motor fluctuations such as the wearing-off phenomenon, remain unclear. One interesting question concerns possible modification of the response to L-DOPA by STN-HFS. We report here the first evidence that STN-HFS interacts in a synergic manner with L-DOPA-induced changes in striatal extracellular dopamine concentration. In DA PL rats, STN-HFS stabilised the L-DOPA-induced increase in striatal dopamine levels during the stimulation period (1 h). These levels remained roughly constant throughout the post-stimulation period (2.5 h), rather than falling back to basal levels, as observed in stimulated intact and non-stimulated rats. Our data, and particularly those for DA PL animals, shed light on the mechanisms underlying the beneficial clinical effects observed in PD patients treated by STN-HFS, including the decrease in L-DOPA requirements and, therefore, in L-DOPA-induced dyskinesia.

Effects of acute L-DOPA injection on striatal extracellular DA, DOPAC and HVA contents

A single acute administration of L-DOPA significantly increased extracellular DA, DOPAC and HVA levels in the striatum of intact and DA PL rats.

Baseline extracellular DA levels in DA PL rats were similar to those in intact animals (Table 1, Fig. 2), suggesting that partial destruction of the SNc probably induces changes in striatal DA content through modifications to DA turnover and reuptake rates. This observation is consistent with published data for rats with similar DA lesions, showing a massive decrease in presynaptic dopaminergic vesicles and an increase in DA turnover (Hefti *et al*., 1980; Robinson and Wishaw, 1988; Zigmond *et al*., 1984; 1989). Moreover, the massive dopaminergic terminal loss in the dorsolateral part of the ipsilateral striatum may also be responsible for a major decrease in presynaptic DA uptake and/or an increase in the ability of dopaminergic neurons to release DA in response to impulse flow (Chritin *et al*., 1996; Zigmond *et al*., 1984; 1989, Dentresangle *et al*., 2001). The mechanisms involved in this compensation of DA levels in

DA PL rats are unlikely to involve a tonic increase in the electrical activity of the dopaminergic neurons. Indeed, more than 80% denervation is required to modify the firing activity of dopaminergic neurons after nigrostriatal lesion (Hollerman and Grace, 1990), suggesting that DA level compensation in DA PL rats is correlated with changes in DA synthesis. Several authors have already shown that DA release is more strongly affected by the inhibition of DA synthesis in 6-OHDA-lesioned rats than in controls (Heffner *et al.,* 1977; Marshall and Teitelbaum, 1973; Snyder *et al.,* 1990). This is consistent with lower levels of amine storage in the dopaminergic terminals and an increase in TH activity (Hefti *et al*., 1985; Stachowiak *et al*., 1987; Snyder *et al*., 1990). Partial lesions of nigrostriatal dopaminergic neurons result in a condition mimicking the early or preclinical phase of PD (Carman *et al*., 1991; Zigmond *et al*., 1984; 1990). The lack of symptoms in patients with this disease before the dopaminergic terminal deficit becomes severe may be a consequence of the passive compensation we observed in DA PL rats. Both dopamine uptake sites (Laihinen *et al*., 1995) and the messenger RNA for the DA transporter (Uhl *et al*., 1994) have been shown to decrease in abundance in clinically diagnosed PD patients. Thus, the consequences of the simultaneous loss of DA release and uptake observed in this animal model may provide direct clues to how normal DA tone is maintained in preclinical subjects.

The L-DOPA-induced increase in striatal extracellular DA levels was more pronounced in DA PL animals (ipsilateral to the lesion) than in intact control rats (Fig 2A; Table 1). In intact striatum, increases in extracellular DA in response to exogenous L-DOPA are more limited because of the lower diffusion coefficient for DA in the extracellular space of the striatum due to the effects of high-affinity DA uptake (Kuhr *et al*., 1986; Kelly *et al*., 1987; Nicholson and Rice, 1991; Garris and Wightman, 1994). It is also possible that DA effectively clears DA synthesised from exogenous L-DOPA from the extracellular fluid of the intact striatum via the DA transporter,{NdT: OK}thereby restricting the diffusion of DA to the microdialysis probes or to the appropriate postsynaptic receptors. This suggests that compensatory mechanisms handle extracellular DA very efficiently in normal conditions, thereby

preventing excessive increases in DA concentration in non-denervated areas, even when L-DOPA concentration is high.

The unrestricted diffusion of DA in the extracellular fluid of the striatum in DA PL animals allows both the detection of extracellular DA at the microdialysis probe and, probably, the activation of dopaminergic receptors (Miller and Abercrombie, 1999). This might account for the marked increase in DA availability after the creation of a partial dopaminergic lesion in the striatum, indicating a disruption of the normal regulation of extrasynaptic DA. These observations confirm previous studies reporting greater selectivity of L-DOPA in DA-lesioned brain areas (therapeutic response) than in normally innervated regions (Abercrombie *et al*., 1990; Maeda *et al*., 1999; Miller and Abercrombie, 1999). Extracellular DOPAC and HVA levels showed a similar marked, longer-lasting response to L-DOPA than to DA (Table 1). However, it is possible that when the rate of DA synthesis exceeds that of DA usage, DA is immediately converted to DOPAC (Arbuthnott *et al.*, 1990; Butcher *et al*., 1988; Fairbrother *et al*., 1990a; 1990b; Westerink and Van Putten, 1987). In this case, a portion of the administered L-DOPA is directly metabolised and therefore has no effect on dopaminergic neurotransmission.

Effect of STN-HFS on L-DOPA-induced increases in striatal extracellular DA, DOPAC and HVA levels

Several clinical studies have reported an excellent clinical outcome of STN stimulation in L-DOPA-responsive forms of PD — patients with selective brain dopaminergic lesions — and a moderate clinical outcome in patients with axial motor symptoms and cognitive impairment known to be less responsive or unresponsive to L-DOPA treatment — development of brain non-dopaminergic lesions in addition to degeneration of the nigrostriatal dopaminergic system (Welter *et al*., 2002; Kleiner-Fisman *et al*., 2003; Pahwa *et al.,* 2005). Chronic bilateral STN stimulation has also been shown to allow the discontinuation of L-DOPA or equivalent treatment or large reductions in daily dose (Moro *et al*., 1999; Fraix *et al*., 2000; Molinuevo *et al*., 2000; Lopiano *et al.,* 2001), resulting in a decrease in L-DOPA-induced

dyskinesia (Krack *et al*., 1997; 1999 ; 2003; Bejjani *et al.*, 2000). These clinical observations suggest that the beneficial effects of STN-HFS on PD symptoms are "L-DOPA-like", but it remains unclear whether the mechanisms of action of STN-HFS and L-DOPA are similar, or even synergic. However, interactions with the dopaminergic system seem to be one key factor in the efficacy of both treatments.

It has been suggested that STN-HFS acts directly on dopaminergic neurons in rodents by increasing their firing rate (Benazzouz *et al*., 2000), TH gene expression in the remaining dopaminergic neurons of the SNc, or by eliciting striatal DA release and the activation of striatal DA, together with striatal TH activity metabolites of PD (Paul *et al.*, 2000; Bruet *et al*., 2001; Meissner *et al*., 2001; 2002; 2003; Henning *et al*., 2007). However, PET studies in humans have given conflicting results, suggesting that an increase in DA transmission cannot be the mechanism underlying the beneficial effects of STN-HFS in advanced PD (Hilker *et al*., 2003; Abosch *et al.*, 2003; Strafella *et al.*, 2003b). However, all these studies in humans were carried out in parkinsonian patients on long-term L-DOPA treatment. The cellular and molecular consequences of such treatment in PD patients, in terms of plasticty within the functional basal ganglia circuitry, remain unknown and it remains unclear how this plasticity interferes with STN-HFS mechanisms or facilitates the alleviation of PD motor symptoms by this treatment. We cannot totally exclude the possibility that STN-HFS mechanisms shunt the dopaminergic system and related circuitries, but this appears unlikely, as the outcome of STN stimulation seems to be correlated with L-DOPA-responsive forms of PD.

We applied STN-HFS to a dopaminergic system activated by L-DOPA in both intact and DA PL rats. The lack of enhancement of the L-DOPA-elicited increase in extracellular DA levels by STN-HFS during the stimulation period suggests that the capacity of the intact or remaining dopaminergic neurons to synthesise and/or release DA has reached its maximum (Table 1 and Fig 2). However, only in DA PL animals did STN-HFS stabilise high striatal DA levels, resulting in an increase in DOPAC and HVA levels during the stimulation and post-stimulation periods. This is consistent with previous push-pull studies showing an increase in DOPAC levels during STN-HFS (100 Hz) with no change in

extracellular DA content (Mintz *et al*., 1986). The equilibrium between the maintenance of high levels of DA and DA degradation resulted in the relative stabilisation/normalisation of DA turnover index (Table 2). This STN-HFS-induced stabilisation of DA turnover index is probably related to the dopaminergic lesion and the capacity of the remaining L-DOPA-activated dopaminergic neurons to respond to STN stimulation directly or indirectly, as this stabilisation is not observed in stimulated intact or non-stimulated DA PL animals, probably as the increase in DA levels is not maintained in these animals (Table 2). These data indicate that STN-HFS interferes with DA turnover, probably by modulating DA uptake and synthesis in DA PL rats, suggesting that adaptive mechanisms are involved in the stabilisation of striatal DA concentrations potentially involved in alleviating L-DOPA-related motor fluctuations, such as the wearing-off phenomenon (Nimura *et al*., 2005). However, the mechanism underlying the stabilisation of striatal DA concentration remains unclear. It may involve the restoration of autoregulation for presynaptic DA release in the striatum. Torstenson *et al*. (1997) demonstrated that the effect of L-DOPA infusion on striatal presynaptic DA activity differs significantly between patients in early and advanced stages of PD. Moreover, Ekesbo *et al.* reported that in mild and stable PD, upregulation of the presynaptic inhibitory feedback system maintains congruity with the dopaminergic system after the administration of antiparkinsonian medication (Ekesbo *et al.*, 1999). The functional tone of the nigrostriatal DA system seems to be regulated at two sites of action: inhibitory autoreceptors located on presynaptic dopaminergic terminals and controlling the synthesis or release of DA, and receptors on the soma or dendrites of these neurons, involved in regulating impulse flow (Bunney *et al*., 1973; Roth, 1979; 1984). This inhibitory feedback regulation decreases with the progression of nigrostriatal degeneration. It seems plausible that STN-HFS alters inputs from the STN to the somatodendritic receptors at the SNc. Autoreceptor function can therefore be restored in the striatum. Alternatively, a relaxation of outputs from the STN may activate the premotor and motor cortices, leading to the attenuation of DA release in the striatum. Repetitive transcranial magnetic stimulation of the human motor cortex has been reported to lead to focal DA release in the ipsilateral

striatum, consistent with a corticostriatal mode of DA release (Strafella *et al*., 2003a). STN-HFS attenuates SNr outputs, overcoming the inhibition of premotor and primary cortices. The decrease in the binding of dopaminergic D2 receptors observed in the known projection area of the disinhibited cortical site indicates that DA release is mediated by a direct effect of the corticostriatal neurons on striatal dopaminergic nerve terminals. Pallidotomy or pallidal stimulation alters the binding of postsynaptic dopaminergic D2 receptors in the striatum, demonstrating an effect of the cortex on the dopaminergic system (Nakajima *et al*., 2003). STN-HFS also increases striatal glutamate levels in both normal and hemiparkinsonian rats, suggesting an increase in the activity of striatal excitatory glutamatergic afferents (Bruet *et al*., 2003). Indeed, the inhibition of basal ganglial output activity by STN-HFS (Benazzouz *et al*., 1995; Windels *et al*., 2000; 2003; 2005; Salin *et al.*, 2002; Boulet *et al*., 2006) may overcome inhibition of the thalamo-cortical pathway (Anderson *et al*., 2003), activating bilateral corticostriatal projections via cortical collaterals and, probably, also the thalamostriatal pathway. This disinhibitory effect is also supported by our recent data showing that STN-HFS increases extracellular GABA levels in the SNr, strongly suggesting that it is involved in inhibiting the BG output structures projecting onto the thalamus (Windels *et al.*, 2000; 2003; 2005; Boulet *et al.*, 2006). A direct corticostriatal influence on striatal dopaminergic terminals could probably account for the spatial selectivity of STN-HFS effects in our study, but we cannot exclude the involvement of other anatomical pathways. Frontal cortical neurons also project onto the SNc (Naito and Kita, 1994), where they can modulate the firing of dopaminergic neurons projecting onto the striatum. Consistent with a previous report, STN-HFS modulated DA concentration in the caudate nucleus after L-DOPA administration, although the degree of change was smaller than that in the putamen (Torstenson *et al*., 1997; Ekesbo *et al*., 1999). The difference between the caudate nucleus and the putamen may reflect the smaller L-DOPA response in the putamen. This may be due to the more severe effects on the dopaminergic terminals of the putamen than on those of the caudate nucleus in PD (Pearson *et al*., 1979; Graybiel *et al.*, 1987; Miller *et al.*, 1997; 1999).

In conclusion, these new neurochemical data suggest that STN-HFS interferes with L-DOPA effects, probably via synergic action, stabilising dopamine levels in the striatum. However, the mechanism underlying this stabilisation remains unclear. Our data show that STN-HFS interferes with DA turnover, probably by modulating DA uptake and synthesis in DA PL rats, suggesting a prolonged smooth DA action and adaptive mechanisms for alleviating L-DOPA-related motor fluctuations, such as the wearing-off phenomenon, shedding light on the mechanisms of STN-HFS in PD.

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Legends

Figure 1:

Photographs of TH-immunostained coronal rat brain sections at the nigral (***A***) and striatal (***B***) levels and of cresyl violet-stained coronal rat brain sections at the striatal (***D***) and subthalamic (***F***) levels in 6-OHDA-lesioned rats. ***C*** and ***E***: Schematic diagrams adapted from the stereotaxic atlas of Paxinos and Watson (1982). Note, on the lesioned side (left), the loss of dopaminergic cells in the lateral part of the SNc (***A***) and the loss of dopaminergic terminals in the dorsolateral part of the striatum (***B***). The location of microdialysis probes in the striatum is schematically represented in ***B*** and visualized in ***D*** on the lesioned side. Note also the correct implantation of the stimulation electrode within the STN (***F***). ***F***, The asterisk indicates the point stimulated. Scale bar, 1 mm.

Ac: Nucleus accumbens; CPu: Caudate putamen; Hi: Hippocampus; LV: Lateral ventricle; SNc: Substantia nigra pars compacta; SN_L: Substantia nigra pars lateralis; SN_R: Substantia nigra pars reticulata; STN: Subthalamic nucleus; VTA: Ventral tegmental area.

Figure 2:

Extracellular DA, DOPAC and HVA levels in the striatum measured in intact and DA PL rats (ipsilateral to the lesion and/or to STN stimulation) following an acute injection of L-DOPA without (A) or with (B) STN-HFS.

The mean concentration ± SEM of the four samples preceding L-DOPA injection was used to determine baseline levels and was set at 100%. The L-DOPA effect represents the mean concentration ± SEM of the last two of the six samples collected following L-DOPA and corresponding to its maximal effect.

In A, the post-L-DOPA values presented correspond to the mean concentration ± SEM of the four (1 h) or six (2.5 h) fractions collected after L-DOPA injection.

In B, post-L-DOPA+STN-HFS values correspond to the mean concentration ± SEM of the four samples collected during 1 h of STN-HFS and after the L-DOPA effect had reached its maximum. Post L-DOPA+Post STN-HFS values correspond to the mean concentration ± SEM of the subsequent 6 samples (2.5 h), collected after the end of STN-HFS.

Note that, in DA PL animals only, STN-HFS stabilised the increase in striatal DA levels induced by L-DOPA and increased levels of DOPAC and HVA during the stimulation and post-stimulation periods.

Results are expressed as a percentage of variation of the baseline value. Each bar corresponds to the mean variation ± SEM calculated from all collected fractions from each animal of each group. White bars = intact rats (A, n=12 ; B, n=13) and grey bars = DA PL rats (A, n=5 ; B, n=8).

*p<0.05, **p<0.001: versus basal values; §: versus L-DOPA effect; †: versus no STN-HFS DA PL rats.

Table 1:

Extracellular levels of DA, DOPAC and HVA (µM) measured in the striatum on the left side of intact and partially SNc-lesioned rats (DA PL) in basal conditions and after acute L-DOPA administration, before (L-DOPA effect), during (post L-DOPA + 1 h STN-HFS) and after (post L-DOPA + post STN-HFS) STN-HFS. The values presented for basal levels and L-DOPA effect periods are averages of the corresponding measures in intact rats, and in DA PL rats. Values corresponding to the "post L-DOPA+ 1 h STN HFS" and the "post L-DOPA+ post STN HFS (t=2.5 h)" periods correspond to values for intact rats subjected to STN-HFS in the part of the table headed "intact", and DA PL rats subjected to STN-HFS in part of the table headed "DA PL". The results are expressed as means ± SEM. ns: not significant.

Table 2:

(DOPAC+HVA) / dopamine ratios for each experimental group.

The results are expressed as means ± SEM. ns: not significant.