Concomitant short- and long-duration response to levodopa in the 6-OHDA-lesioned rat: a behavioural and molecular study

C. Marin,1 E. Aguilar,1 G. Mengod,2 R. Cortés2 and J. A. Obeso3
1Laboratori de Neurologia Experimental, Àrea de Neurociències, Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain
2Departament de Neuroquimica, Institut d’Investigacions Biomèdiques de Barcelona, CSIC-IDIBAPS, Barcelona, Spain
3Department of Neurology and Neurosurgery, Neuroscience Center, Clínica Universitaria and Medical School, University of Navarra and CIMA, Pamplona, Spain

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Abstract
The long-duration response (LDR) is a sustained improvement in parkinsonism due to chronic levodopa therapy and lasts after discontinuation of treatment. We have investigated the molecular changes that underlie the LDR in rats with a unilateral 6-hydroxydopamine (6-OHDA) lesion. Animals were treated for 22 days with levodopa or saline. Forelimb akinesia was evaluated prior and following a test dose of levodopa. Rotation behaviour was weekly evaluated. Levodopa induced an improvement in the parkinsonian limb akinesia that lasted for 48 h after withdrawal. A shortening in the duration of rotational behaviour was observed. After 3 days of washout, levodopa treatment maintained elevated striatal preproenkephalin mRNA expression, also inducing an increase in preprodynorphin (PDyn) and dopamine D-3 receptor mRNAs, but without any modification of the adenosine A2A mRNA expression induced by 6-OHDA. Levodopa reversed the lesion-induced increase in the expression of cytochrome oxidase mRNA in the subthalamic nucleus and glutamate decarboxylase mRNA in the pars reticulata of the substantia nigra. After 7 days of levodopa washout, the molecular markers show a decline in the basal ganglia evolving towards the parkinsonian state, being statistically significant for the striatal PDyn mRNA. This study characterizes the concomitant presence of the short-duration response and LDR to levodopa in the 6-OHDA model of parkinsonism and shows that the molecular changes induced by levodopa in the basal ganglia are not permanent and that this reversal after levedopa washout may be responsible for the gradual motor deterioration that characterize the LDR.

Introduction
The motor response to levodopa in Parkinson’s disease (PD) is comprised of two main components: the long-duration response (LDR) and the short-duration response (SDR) (Muenter & Tyce, 1971). The SDR is characterized by a short-lasting motor improvement typically lasting 3–4 h following a single dose of levodopa. The SDR is the basis for the clinical phenomenon of ‘wearing-off’ that parallels the rise and fall of levodopa plasma levels. The LDR is a sustained motor improvement that takes days to build up and lasts for many hours to days after levodopa discontinuation (Nutt et al., 1995; Quattrone et al., 1995; Zappia et al., 1997; Parkinson Study Group, 2004). A recent study (the ELLDOPA trial) in de novo patients has shown that the LDR may last actually up to several weeks after cessation of levodopa treatment (Parkinson Study Group, 2004). This suggests a very potent capacity of levodopa to restore the physiology of the basal ganglia and enhance mechanisms compensating for the dopaminergic deficit in PD.

The pathophysiological mechanisms underlying the LDR are still unknown. The association with levodopa treatment suggested a presynaptic mechanism, i.e. residual dopaminergic neurons could take up and decarboxylate exogenous levodopa, store and release dopamine (DA) over a prolonged period (Zappia et al., 1997). On the other hand, LDR is also observed after treatment with DA agonists such as apomorphine (Nutt & Carter, 2000), ropinirole (Barbato et al., 1997) and lisuride (Stocchi et al., 2001), indicating a role of postsynaptic mechanisms.

In animal models of PD, differential alterations in the expression of striatal neuropeptides mRNAs correlate with some of the changes in neuronal activity within the basal ganglia in the DA-depleted state and with the motor responses induced by levodopa. Thus, striatal levels of preproenkephalin (PPE) mRNA in the indirect pathway are increased in the 6-hydroxydopamine (6-OHDA)-lesioned rats (Zeng et al., 1995; Henry et al., 1998, 1999; Ravenscroft et al., 2004) and in MPTP-treated monkeys (Jolkkonen et al., 1995; Morissette et al., 1997; Bezard et al., 2001). However, preprodynorphin (PDyn) mRNA expression in the direct pathway is decreased in 6-OHDA-lesioned rats (Henry et al., 1999; Ravenscroft et al., 2004) and in MPTP monkeys (Jolkkonen et al., 1995). It has been widely reported that chronic treatment with levodopa reverses the decrease in striatal PDyn mRNA (Andersson et al., 1999; Henry et al., 1999; Carta et al., 2002; Ravenscroft et al., 2004) and may increase PPE mRNA over basal levels (Morissette et al., 1997, 1999; Ravenscroft et al., 2004; Chen et al., 2005). In addition, levodopa administration in the 6-OHDA-lesioned rat normalizes the increased expression of cytochrome oxidase (CO) mRNA in the subthalamic nucleus (STN) (Vila et al.,
Materials and methods

6-OHDA lesions and protocol of treatments
Male Sprague–Dawley rats weighing 220–250 g were housed on a 12 h light : dark cycle with free access to food and water. All the experiments were performed in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals, and with the approval of the Ethical Committees of the University of Barcelona and the Hospital Clinic of Barcelona. Under sodium pentobarbital anaesthesia, the rats were placed in a stereotaxic frame with the incisor bar positioned 4.5 mm below the interaural line. Each animal received a 6-OHDA injection (8 μg in 4 μL of saline with 0.02% ascorbate over 8 min) into the left medial forebrain bundle by means of a Harvard infusion pump. Stereotaxic injections were placed 4.0 mm anterior to the interaural line, 1.3 mm lateral to the midline and 8.4 mm ventral to the surface of the skull, according to the atlas of Paxinos & Watson (1986).

6-OHDA-lesioned rats received a 22-day treatment with levodopa methyl ester (25 mg/kg with 6.25 mg/kg of benserazide, i.p., twice a day). The two drugs were dissolved in saline. Control animals were treated with saline.

Experimental design
LDR induced by levodopa was investigated before and after a dose test of levodopa (6 mg/kg) in 6-OHDA-lesioned rats during and until 7 days after levodopa chronic treatment. The molecular changes associated with the LDR were investigated in another group of animals chronically treated with levodopa following the same protocol as for the behavioural studies but without receiving the dose test of levodopa. To this aim, animals were killed 3 and 7 days after discontinuation of the 22-day period of levodopa treatment.

Rotational screening
For the measurement of rotational behaviour, rats were placed in circular cages and tethered to an automated rotometer. The number of complete (360°) turns made during each 5-min period was recorded by computer. Rats were allowed 15 min to habituate to the rotometer before the administration of drugs. Following a 3-week recovery period, rats exhibiting a vigorous rotational response (> 100 total turns) to apomorphine (0.05 mg/kg, s.c.) were selected for further study. It has previously been demonstrated that rats meeting this criterion have a greater than 95% depletion of striatal DA (Papa et al., 1994).

The duration of rotation was measured as the efficacy half-time, i.e. time elapsing from the first 5-min interval when turning exceeded the half-maximal rate to the first interval when it fell below the half-maximal rate. Rotational behaviour was weekly measured.

Forelimb akinesia – cylinder test
To assess forelimb akinesia the cylinder test was used as previously described (Schallert et al., 2000). Rats were placed individually in a circular cylinder and the number of supporting well contacts that were carried out with each of the right (parkinsonian) and left forelimbs was counted for a period of 5 min prior and 45 min after the administration of a test dose of levodopa (6 mg/kg). No habituation to the cylinder prior to testing was allowed. Forelimb akinesia was evaluated before and after 6-OHDA lesion, weekly during levodopa treatment and 4, 2, 5 and 7 days after levodopa withdrawal.

Tissue collection
Three or seven days after the last levodopa or saline administration, rats were killed under an overdose of pentobarbital anaesthesia. Brains were quickly removed from the skull and then frozen on dry ice and kept at −80 °C until they were cut on a microtome-cryostat. Coronal 14-μm-thick sections were collected through the striatum, thaw-mounted onto 3-amino-propyltriethoxysilane (APTS)-coated slides, and kept at −40 °C until used.

DA transporter (DAT) immunohistochemistry
Striatal sections were thawed and dried at room temperature, and fixed with acetone for 10 min at 4 °C. The sections were rinsed in phosphate-buffered saline (PBS), pH 7.4, twice, 5 min each, and immersed in 0.3% hydrogen peroxide in PBS for 10 min to block the endogenous peroxidase. At this point, sections were rinsed again in PBS and incubated with horse serum with 0.1% Triton X-100 for 20 min. Sections were incubated overnight at 4 °C with mouse anti-DAT monoclonal antibody at a dilution 1 : 500 in PBS. Sections were rinsed twice in PBS, 5 min each, and ImmunoPure Ultra-Sensitive ABC peroxidase staining kit was used to carry out the ABC staining method. By so doing, sections were incubated with biotinylated horse anti-mouse IgG for 30 min, followed by two rinses in PBS, and then incubated with avidin-biotinylated peroxidase complex for 30 min more. Finally, sections were rinsed in PBS and incubated with 3,3′-diaminobenzidine and 0.01% hydrogen peroxide for 15 min. Slides were washed with PBS, dehydrated in ascending alcohol concentrations, cleared in xylene and coverslipped in DPX-EXLI mounting medium.

In situ hybridization histochemistry
The oligonucleotides used were complementary to the following base sequences (GeneBank accession number in brackets): PDyn, bases 607–654 [NM_019374]; PPE, bases 513–542 [K02807]; CO subunit I, bases 644–688 [S79304]; adenosine A2A receptor, bases 285–329 [NM_00675]; GAD67, bases 191–235 and 1600–1653 [NM_01700]; DA D3 receptors, bases 85–132, 1144–1191 and 1381–1426
They were synthesized and high-performance liquid chromatography purified by Amersham Pharmacia Biotech (UK) and by Isogen Bioscience BV (Maarsden, the Netherlands). The oligonucleotides were labelled at their 3’-end by using [\textsuperscript{33}P]dATP (3000 Ci/mmol, New England Nuclear, Boston, MA, USA) and terminal deoxynucleotidyl-transferase (Oncogene Research Products, San Diego, CA, USA), and purified using QIAquick Nucleotide Removal Kit (QIAGEN GmbH, Hilden, Germany).

For in situ hybridization, frozen tissue striatal sections were brought to room temperature, air-dried and fixed for 20 min in 4% paraformaldehyde in PBS (1 × PBS: in mM: KCl, 2.6; KH$_2$PO$_4$, 1.4; NaCl, 136; Na$_2$HPO$_4$, 8), washed once in 3 × PBS, twice in 1 × PBS, 5 min each, and incubated in a freshly prepared solution of predigested pronase at a final concentration of 24 U/mL in 50 mM Tris–HCl pH 7.5, 5 mM EDTA. The enzymatic activity was stopped by immersion for 30 s in 2 mg/mL glycine in 1 × PBS. Tissues were finally rinsed in 1 × PBS and dehydrated through a graded series of ethanol. For hybridization, radioactively labelled probes were diluted to a final concentration of $10^7$ cpm/mL in a solution containing 50% formamide, 4 × standard sodium citrate (SSC) (1 × SSC: in mM: NaCl, 150; sodium citrate, 15), 1 × Denhardt’s solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 10% dextran sulphate, 1% sarkosyl, 20 mM phosphate buffer pH 7.0, 250 µg/mL yeast tRNA and 500 µg/mL salmon sperm DNA. Tissues were covered with 100 µL of the hybridization solution and overlaid with Nescofilm (Bando Chemical, Kobe, Japan) coverslips to prevent evaporation. Sections were incubated in humid boxes overnight at 42 °C and then washed four times (45 min each) in (in mM): NaCl, 600; Tris–HCl, 20, pH 7.5; EDTA, 1; at 60 °C. Hybridized sections were exposed to BIOMAX-MR film (Kodak) for 1–5 days depending on the probe used at ~70 °C with intensifying screens.

The specificity of the nucleotide hybridization signals was assessed as follows. For a given oligonucleotide probe the presence of a 50-fold excess of the same unlabelled oligonucleotide in the hybridization buffer resulted in the abolishment of the specific hybridization signal (data not shown). The thermal stability of the hybrids was examined by washing a series of consecutive hybridized sections at increasing temperatures. Specific hybridization signals were still present in sections washed at 70 °C, but they were completely absent from sections washed at 80 °C. No such decrease was observed in the background levels of the signal (data not shown).

In order to evaluate specific topographical molecular changes, the striatum were divided into four portions for the mRNAs measurements, including the dorsolateral, ventrolateral, dorsomedial and ventromedial striatum. The average densities of the mRNAs in different brain regions were evaluated semi-quantitatively, as relative optical densities, on film autoradiograms with the aid of an image analysis system (MCID M4, Imaging Research, St Catherines, Ontario, Canada).

Hybridized tissue section images from film autoradiograms were photographed with a digital camera (DXM1200 F, Nikon) and ACT-1 Nikon Software using a Wild makroscope M-420. Figures were prepared for publication using Adobe Photoshop software (Adobe Software, San Jose, CA, USA). Contrast and brightness of images were the only variables we adjusted digitally. For anatomical reference, sections close to those used were stained with Cresyl violet.

**Statistics**

Data were analysed by analysis for variance (ANOVA) followed by Dunnett’s t-test for multiple comparisons or paired/unpaired Student’s t-test when required. The level of statistical significance was set at $P < 0.05$ for all analyses.

**Results**

**Characterization of the unilateral 6-OHDA lesion**

Rats with a complete 6-OHDA lesion exhibited a rotational response to apomorphine injection (> 100 rotations). A quantitative analysis showed an absence of DAT-immunoreactivity in the ipsilateral striatum (Fig. 1). No significant differences in the degree of

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**Fig. 1.** Representative dopamine transporter (DAT) immunohistochemistry from 14-µm coronal sections of the rostral striatum. Sections are from rats receiving 6-OHDA injection in the left forebrain bundle, then treated for 22 days with: (A) saline; (B) levodopa (25 mg/kg) and killed 3 days after last administration; and (C) levodopa (25 mg/kg) and killed 7 days after last administration. Note the significant absence of DAT-immunoreactivity in the lesioned side regardless of the drug treatment or washout period.

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dopaminergic denervation were observed among the groups of rats that received levodopa or saline administration (Fig. 1).

**Effects of chronic administration of levodopa on rotational behaviour**

The total number of rotations and the peak of maximal rotation induced by levodopa increased after 22 days of treatment ($P < 0.05$) (Table 1). The duration of the motor response to levodopa significantly shortened (22%) by the 22nd day of treatment in animals receiving two daily administrations of levodopa ($P < 0.01$) (Fig. 2).

**Effects of chronic administration of levodopa on forelimb akinesia**

Prior to 6-OHDA lesion, the proportion of wall contacts performed with the right paw amounted to 55% of the total in all animals. This percentage of wall contacts with the right (parkinsonian) paw was minimal 3 weeks after 6-OHDA lesion ($P < 0.01$) (Fig. 3).

The limb asymmetry test measured before the administration of the test dose of levodopa did not improve throughout the experiment (Fig. 3A). However, 45 min after the test dose of levodopa (6 mg/kg), a significant improvement in limb use asymmetry was observed in the group of animals chronically treated with levodopa (25 mg/kg) (Fig. 3B). This effect reached similar percentages of wall contacts than the ones achieved by the same limb prior to 6-OHDA lesion. The benefit lasted for several days, although the increased limb activity was only significant up to 48 h after levodopa treatment withdrawal (Fig. 3B).

**Striatal PPE mRNA expression**

A significant 46% increase in PPE mRNA levels in the lesioned striatum was caused by the 6-OHDA lesion in the saline-treated animals compared with the intact striatum ($P < 0.01$) (Fig. 4A). After 3 days of washout, rats that received treatment with levodopa showed a 76% increase in striatal PPE levels with respect to the intact striatum ($P < 0.01$). The levels of PPE mRNA remained significantly higher in the lesioned striatum compared with the intact striatum after 7 days washout, with a tendency to decline by 11% (Fig. 4A). PPE mRNA expression levels were not modified by levodopa in the intact striatum of any group (Fig. 4A). Similar results have been found when topographical analysis was performed in the four sections of the striatum (Table 2).

**Striatal PDyn mRNA expression**

Striatal PDyn mRNA levels were significantly reduced (by 24%) in the 6-OHDA-lesioned striatum ($P < 0.01$) in the saline-treated animals compared with the intact striatum (Fig. 4B). Rats that received chronic intermittent treatment with levodopa showed a significant increase in PDyn mRNA in the lesioned striatum by 118% and 72% at 3 days and 7 days, respectively, after discontinuation of levodopa treatment ($P < 0.01$) in comparison with saline-treated rats. However, the levels of PDyn mRNA were significantly reduced by 21% in animals killed 7 days after the last administration of levodopa in comparison with animals killed after 3 days washout ($P < 0.05$) (Fig. 4B). Similar results were found when topographical analysis was performed in the four sections of the striatum (Table 2). There were no significant changes in PDyn mRNA expression levels in the intact side in any group.

**Striatal adenosine A2A mRNA expression**

A$_{2A}$ mRNA expression was not modified by the 6-OHDA lesion compared with the intact striatum (Fig. 5A). Chronic intermittent treatment with levodopa did not significantly change the striatal levels of A$_{2A}$ mRNA in the lesioned striatum. No differences were observed after a 3- or 7-day washout period after levodopa treatment (Fig. 5A). Similar results have been found when topographical analysis was performed in the four sections of the striatum (Table 2). There were no significant changes in PDyn mRNA expression levels in the intact side in any group.

**Striatal DA D3 receptor mRNA expression**

DA D3 receptor mRNA expression was not modified by the 6-OHDA lesion compared with the intact striatum (Fig. 5B). After 3 days of washout, rats that received chronic intermittent treatment with levodopa showed a small (8%) but significant increase in DA D3 mRNA in the lesioned striatum in comparison with saline-treated rats ($P < 0.05$) and with the intact side ($P < 0.01$). In animals killed 7 days after the last administration of levodopa, the levels of DA D3 receptor mRNA were significantly higher than the intact side ($P < 0.05$), but not in comparison with saline-treated animals (Fig. 5B), although these levels are still increased in comparison with
saline-treated animals ($P < 0.01$). There were no significant changes in DA D3 mRNA expression levels in the intact side in any group. Similar results have been found when topographical analysis was performed in the four sections of the striatum, being more significant in the lateral striatum (Table 2).

**Subthalamic CO mRNA expression**

Unilateral 6-OHDA lesion induced a significant increase (10%) in CO mRNA expression in the ipsilateral STN ($P < 0.05$) (Fig. 6A). After 3 days of washout, rats that received chronic intermittent treatment with levodopa showed a significant decrease (27%) in CO mRNA in the STN in comparison with saline-treated rats ($P < 0.01$). This effect was noticeable as the levels were normalized in comparison with the intact side in the same group. In animals killed 7 days after the last administration of levodopa, the levels of CO mRNA in the STN ipsilateral to the lesion showed an increase by 22%, that was not statistically significant (Fig. 6A). The expression of CO mRNA in the STN in rats killed 7 days after the last levodopa administration fell in between the saline-treated group (i.e. parkinsonian state) and the levodopa-treated group with 3 days washout. Thus, there were no

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**Fig. 3.** Long-duration response (LDR) to levodopa. (A) Baseline forelimb akinesia in unilateral 6-hydroxydopamine (6-OHDA)-lesioned rats chronically treated with levodopa (25 mg/kg plus benserazide 6.25 mg/kg, twice a day, i.p., $n = 9$) or saline. (B) Forelimb akinesia evaluated 45 min after a levodopa dose-test (6 mg/kg, i.p.) in unilateral 6-OHDA-lesioned rats chronically treated with levodopa + vehicle (25 mg/kg plus benserazide 6.25 mg/kg, twice a day, i.p., $n = 9$). $$$P < 0.01$ vs. before 6-OHDA; $^*P < 0.05,$ $^{**}P < 0.01$ vs. 22 days post–OHDA.
significant differences in comparison with either group (Fig. 6A).
There were no significant changes in CO mRNA expression levels in
the STN contralateral to the lesion.

Nigral GAD mRNA expression
Unilateral 6-OHDA lesion induced a significant increase (17%) in
GAD mRNA expression in the ipsilateral SNr (P < 0.05) (Fig. 6B).
After 3 days of washout, rats that received chronic intermittent
treatment with levodopa showed a significant 18% decrease in GAD
mRNA in the ipsilateral SNr in comparison with saline-treated rats
(P < 0.01). This produced levels that were normalized in comparison
with the intact side. In animals killed 7 days after the last adminis-
tration of levodopa, the levels of GAD mRNA in the ipsilateral SNr
showed a tendency to increase by 8%. The expression of GAD mRNA
in the SNr in rats killed 7 days after the last levodopa administration
was not significant in comparison with either the animals with 3 days
washout period or with the increase observed in the saline-treated
group (Fig. 6B). This evolution mimicked the one described above for

Fig. 4. Striatal preproenkephalin (PPE) (A) and preprodynorphin (PDyn) (B) mRNAs expression in 6-OHDA-lesioned rats chronically treated with levodopa
(25 mg/kg plus benserazide 6.25 mg/kg; twice a day, n = 5) and killed 3 or 7 days after discontinuation of treatment. Control animals were treated with saline.
Left: values are expressed as mean ± SEM. *P < 0.05, **P < 0.01 vs. the corresponding intact side; ##P < 0.01 vs. saline-treated animals; + P < 0.05 vs. levodopa
3 days washout. Right: representative film autoradiograms of coronal brain sections (14 μm) showing striatal PPE and PDyn mRNAs labelling in all treatment
groups.
**Discussion**

We have found that the motor improvement induced by daily levodopa administration in the 6-OHDA rat model had a short lasting effect but it persists partially for some 2–5 days after the last dose. This in effect represents a combination of a SDR and LDR to levodopa, a phenomenon that to the best of our knowledge had not been described before in the 6-OHDA rat model. In these animals, the SDR to levodopa manifested as rotational behaviour consists of an immediate response elicited by the dose of levodopa that increased in magnitude through the treatment period but decreased in duration, resembling the ‘wearing-off’ fluctuation in parkinsonian patients (Papa et al., 1996). This could involve a slowly turning over pool of levodopa or, more likely, of DA produced by residual dopaminergic neurons that could take up and decarboxylate exogenous levodopa (Nutt & Holford, 1996). However, a LDR can be produced in the *de novo* patients with the DA agonist apomorphine (Nutt & Carter, 2000), ropinirole (Barbato et al., 1997) and lisuride (Stoocchi et al., 2001), suggesting that the LDR could be mediated postsynthetically. Presynaptic storage of DA cannot explain the LDR induced by the direct DA agonists as they cannot be stored in the presynaptic terminals. Moreover, in patients with PD it has been observed that the LDR to levodopa is larger in the more affected arm (Nutt et al., 2000), indicating that the LDR is not dependent on residual DA terminals and the storage of DA. According with such clinical evidence, the LDR to levodopa seems to require the activation of postsynaptic DA receptor and may be related to long-lasting changes in synaptic transmission and downstream basal ganglia mechanisms. Dopaminergic denervation induces complex changes in striatal genes expression that occur in both the ‘direct’ (D1-mediated) and ‘indirect’ (D2-mediated) striatal output pathways. Persistent changes, more than 1 year, in striatal gene expression produced by chronic dopaminergic denervation in the 6-OHDA model have been described for opioid precursor gene expression (Westin et al., 2001). However, the possible contribution of molecular changes to the LDR induced by levodopa and whether levodopa-induced changes in striatal gene expression are long-lasting or rather represent an initial adaptive response to the treatment is not defined. Our results show that the molecular changes induced by levodopa are by and large reversible. Moreover, the return of some molecular markers, and the tendency of others, to levels seen in the parkinsonian state may be related with the progressive loss of the LDR induced by levodopa.

In agreement with most previous studies, we found that long-term levodopa treatment did not reverse the PPE mRNA upregulation induced by the 6-OHDA lesion (Salin et al., 1997; Westin et al., 2001; Perier et al., 2003; Bové et al., 2006). Moreover, in animals killed after 3 days levodopa withdrawal the tendency, although not statistically significant, was toward increased PPE mRNA expression. This

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**Table 2. Expression of the molecular markers in the four sections of the striatum**

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Levodopa 3 days washout</th>
<th>Levodopa 7 days washout</th>
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<tr>
<td></td>
<td>Intact</td>
<td>Lesioned</td>
<td>Intact</td>
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<tr>
<td><strong>PPE mRNA</strong></td>
<td></td>
<td></td>
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<tr>
<td>DL striatum</td>
<td>0.261 ± 0.021</td>
<td>0.37 ± 0.003**</td>
<td>0.254 ± 0.007</td>
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<td>0.387 ± 0.03**</td>
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<td>0.289 ± 0.002</td>
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<td>0.279 ± 0.007</td>
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<td>VM striatum</td>
<td>0.254 ± 0.012</td>
<td>0.375 ± 0.03**</td>
<td>0.245 ± 0.008</td>
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<td><strong>PDyn mRNA</strong></td>
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<td></td>
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<tr>
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<td>0.177 ± 0.011</td>
<td>0.125 ± 0.005**</td>
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<td>0.16 ± 0.021**</td>
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<td>VM striatum</td>
<td>0.259 ± 0.02</td>
<td>0.184 ± 0.013**</td>
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<td>0.126 ± 0.002</td>
<td>0.131 ± 0.005</td>
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Values are expressed as mean ± SEM. *P < 0.05, **P < 0.01 vs. intact side; ##P < 0.05, ###P < 0.01 vs. saline-treated animals; + P < 0.05 vs. levodopa 3 days washout.

the SNr contralateral to the lesion.
tendency to increase PPE mRNA levels was lost in the animals killed 7 days after the end of levodopa treatment. This effect was observed both when considering the whole striatal tissue volume and in each one of the four sections of the striatum individually measured.

As previously shown, 6-OHDA lesion induced a decrease in striatal PDyn mRNA expression (Duty et al., 1998; Gerfen et al., 1990, 1991; Henry et al., 1999). As expected, levodopa treatment significantly increased striatal PDyn expression as observed after 3 days of washout (Cenci et al., 1998; Andersson et al., 1999; Bordet et al., 2000; Van de Witte et al., 2002; Winkler et al., 2002; St-Hilaire et al., 2003). However, after 7 days of washout the increment in striatal PDyn mRNA expression was significantly lower than that observed at 3 days washout. These results are in agreement with a previous report by Andersson et al. (2003), who observed a progressive decline in deltaFosB and PDyn mRNA levels after discontinuation of chronic levodopa.

Adenosine A2A receptors have been localized to γ-aminobutyric acid (GABA)ergic striatopallidal neurons (Hettinger et al., 2001). The lack of modification of striatal A2A mRNA expression after 6-OHDA lesion is in agreement with previous reports (Morelli et al., 1994; Kaelin-Lang et al., 2000; Tomiyama et al., 2004; Bové et al., 2006). No further changes were observed after levodopa treatment in either of

Fig. 5. Striatal adenosine A2A (A) and DA D3 (B) mRNAs expression in 6-OHDA-lesioned rats chronically treated with levodopa (25 mg/kg plus benserazide 6.25 mg/kg, twice a day, n = 5) and killed 3 or 7 days after discontinuation of treatment. Control animals were treated with saline. Left: values are expressed as mean ± SEM. *P < 0.05, **P < 0.01 vs. the corresponding intact side; #P < 0.05 vs. saline-treated animals. Right: representative film autoradiograms of coronal brain sections (14 µm) showing striatal A2A and DA D3 mRNAs labelling in all treatment groups.
the washout periods studied according with several publications (Jenner, 2000; Bovè et al., 2006), but in contrast with others (Tomiyama et al., 2004). Controversial results have been previously described, in relation to mRNA expression of A2A receptors arising from the fact that methodological issues might be involved such as the dose of levodopa, the duration of the treatment and the time lapsed between the last administration of the drugs and death (Tomiyama et al., 2004).

The implication of DA D3 receptor subtype in the development of both behavioural and molecular adaptations induced by repeated levodopa treatment in 6-OHDA hemiparkinsonian rats has been reported over the last years (Bordet et al., 1997; Van Kampen & Stoessl, 2000, 2003; Aubert et al., 2005). D3 receptor mRNA is expressed in the striatal medium spiny neurons of the direct pathway, that is, those that express the D1 receptor (Bordet et al., 2000; Aubert et al., 2005). As previously reported (Bordet et al., 1997; Bezard et al., 2003; Guigoni et al., 2005; St-Hilaire et al., 2005), levodopa treatment increased the levels of striatal D3 mRNA as observed in the group of animals after 3 days washout. After 7 days of washout the increase in striatal D3 mRNA persisted without any modification.
Regarding the molecular activity in other basal ganglia nuclei, levodopa treatment reversed the 6-OHDA lesion-induced overexpression of CO mRNA in the STN (Vila et al., 1999; Perier et al., 2003) in the group of animals studied after 3 days of washout. After 7 days of washout, a tendency to increase the levels of mRNA CO in the STN was observed. Similar results were obtained in the ipsilateral SNr where levodopa treatment significantly reduced the increase in nigral GAD67 mRNA induced by 6-OHDA (Vila et al., 1999; Perier et al., 2003), as observed in the group of animals after 3 days of washout. After 7 days of washout a tendency to increase these levels was also achieved.

All these findings indicate that the output of the basal ganglia persists in a state closer to the levodopa-treated situation for a few days after levodopa withdrawal. Later, all the molecular changes, except striatal DA D3 receptor expression, tend to return to the parkinsonian state levels correlating with the loss of LDR. Such a slow shift towards the parkinsonian state could sustain the LDR.

In summary, the present study is the first to report the presence of concomitant SDR and LDR to levodopa in the unilateral 6-OHDA model of parkinsonism. This new model may provide a useful tool to study the molecular mechanisms involved in the LDR to levodopa. Our results confirm that long-term levodopa treatment in 6-OHDA-lesioned rats creates a new functional state in the basal ganglia different from the normal physiological and the DA-deafferented states that correlate with its antiparkinsonian action, as previously reported (Salin et al., 1997; Henry et al., 1999; Nielsen & Soghomonian, 2003, 2004). Interestingly, the novelty of our results relies in the observation that this new functional state induced by levodopa treatment evolves slowly towards the parkinsonian state as observed in rats with a 7-day washout period. This slow return to the parkinsonian state might be the underlying mechanism of the LDR to levodopa after treatment discontinuation.

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Abbreviations

6-OHDA, 6-hydroxydopamine; CO, cytochrome oxidase; DA, dopamine; DAT, dopamine transporter; GAD, glutamate decarboxylase; LDR, long-duration response; PBS, phosphate-buffered saline; PD, Parkinson’s disease; PDyn, preprodynorphin; PPE, preproenkephalin; SDR, short-duration response; SNr, pars reticulata of the substantia nigra; SSC, standard sodium citrate; STN, subthalamic nucleus.

References


