Regular article

Entacapone potentiates the long-duration response but does not normalize levodopa-induced molecular changes

C. Marin[1,4], E. Aguilar[1], G. Mengod[2,4], R. Cortés[2,4], M.C. Rodríguez-Oroz[3,4], J. A. Obeso[3,4]

[1]Laboratori de Neurologia Experimental, Àrea de Neurociències, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain

[2]Departament de Neuroquímica i Neurofarmacologia, Institut d'Investigacions Biomèdiques de Barcelona, CSIC-IDIBAPS, Barcelona, Spain

[3]Department of Neurology and Neurosurgery, Neuroscience Center, Clínica Universitaria and Medical School, University of Navarra and CIMA, Pamplona, Spain.

[4]Centro de Investigación en Redes sobre Enfermedades Neurodegenerativas (CIBERNED), Spain

Address for correspondence:

Dr. Concepció Marin

Laboratori de Neurologia
Servei de Neurologia
Hospital Clínic
Villarroel 170
08036 Barcelona
Spain
Ph: 34-93-2275400 ext. 2908
Fax: 34-93-2275783
e-mail: cmarin@clinic.ub.es

Abstract

Coadministration of entacapone with levodopa attenuates motor complications in experimental models of Parkinson's disease. The mechanisms underlying entacapone effects are unknown. We investigated the effect of entacapone, on: long duration response (LDR) to levodopa, levodopa-induced postsynaptic pharmacodynamic mechanisms and molecular changes in hemiparkinsonian rats. 6-hydroxydopamine-unilaterally lesion rats were treated with levodopa (25 mg /kg)+vehicle; levodopa+entacapone (30 mg/kg) or saline, twice daily for 22 days. The LDR and the apomorphine-induced rotations were measured. *In situ* hybridization was performed measuring the expression of striatal preproenkephalin, preprodynorphin and dopamine D-3 receptors mRNAs, subthalamic cytochrome oxidase mRNA and nigral glutamic acid decarboxylase mRNA. Entacapone potentiated the LDR but did no modify neither the apomorphine-induced rotational behavior nor the molecular changes. Our results suggest that the effects of entacapone on levodopa-induced motor response are no mediated by postsynaptic mechanisms and that administration of entacapone is not able to normalize the molecular alterations induced by levodopa in the basal ganglia.

Keywords: Parkinson's disease, levodopa, entacapone. 6-hydroxydopamine, preproenkephalin, preprodynorphin

Introduction

Under physiological circumstances, extra-synaptic striatal dopamine (DA) levels are relatively constant and DA receptors are stimulated in a relatively continuous manner (Abercrombie *et al.* 1990; Floresco *et al.* 2003; Venton *et al.* 2003). In Parkinson's disease (PD), there is a loss of dopaminergic neurons and terminals and striatal DA levels are increasingly dependent on the peripheral availability of levodopa (Abercrombie *et al*. 1990; Miller and Abercrombie 1999; de la Fuente-Fernandez *et al.* 2004). Therefore, in advanced PD the fluctuations in plasma levels of short-acting dopaminergic agents, such as levodopa, lead to alternating high and low levels of activation of striatal DA receptors. This "pulsatile" pattern of receptor stimulation is a function of both, disease severity (Bedard *et al.* 1986; Pearce *et al*. 1998, 2001; Jenner 2000) and the short half-life of the dopaminergic agent employed (Bedard *et al.* 1986; Nutt 1990; Pearce *et al.* 1998; Olanow and Obeso 2000; Olanow *et al*. 2006). Sustained evidence has been accumulated indicating that levodopa-related motor complications in PD are associated with non-physiological, intermittent or pulsatile stimulation of striatal DA receptors (Chase *et al.* 1989, 1994; Juncos *et al.* 1989; Blanchet *et al.* 1995; Grondin *et al.* 1996; Stocchi and Olanow 2004; Olanow *et al*. 2006; Fabbrini *et al.* 2007).

This concept suggests that a more continuous stimulation of DA receptors may ameliorate or even reduce the development of motor complications. The former has been shown in PD (Quinn *et al.* 1984; Obeso *et al.* 1986; Mouradian *et al.* 1990; Nutt 1990; Olanow *et al*. 2006; Nyholm 2007) and the latter in experimental parkinsonism (Bedard *et al.* 1986; Blanchet *et al.* 1995; Grondin *et al.* 1996; Pearce *et al.* 1998; Maratos *et al.* 2001; Jenner 2004; Smith *et al.* 2005; Nyholm 2007). The concept of continuous dopaminergic stimulation (CDS) is supported by a wealth of experimental data showing that the dopaminergic system exerts a dual (tonic and phasic) effect on the striatum (Grace 1991; Floresco *et al*. 2003). In patients with PD, clinical pharmacological studies also support the therapeutical concept of CDS (Chase *et al.* 1989, Obeso *et al.* 1994, 2000; Olanow and Obeso 2000; Olanow et al., 2006).

There is evidence suggesting that pulsatile striatal stimulation induce postsynaptic pharmacodynamic mechanisms which result in altered regulation of striatal genes and proteins, which ultimately leads to abnormal neuronal firing patterns in the striatopallidal circuitries (Herrero *et al.* 1995; Jolkkonen *et al.* 1995; Morissette *et al.* 1997, 1999; Olanow and Obeso 2000; Aubert *et al*. 2005; Gardoni *et al.* 2006; Bychkov *et al.* 2007). In more detail, chronic levodopa or dopamine agonist treatment elevates the expression of the striatal enkephalin precursor preproenkephalin (PPE), the opioid peptide expressed in the striatal neurons that project to the pars externa of the globus pallidus (GPe), above the levels reached after DA denervation alone (Gerfen *et al.* 1990; Herrero *et al*. 1995; Henry *et al.* 1999; Ravenscroft *et al*. 2004; Chen *et al.* 2005). Moreover, a significant correlation has been found between levodopa-induced complications and increased striatal levels of preprodynorphin (PDyn) mRNA, the opioid peptide expressed in the striatal neurons that project directly to the output structures of the basal ganglia, in a rodent model

of PD (Cenci *et al.* 1998; Andersson *et al*. 1999; Carta *et al.* 2002; Winkler *et al.* 2002; Ravenscroft *et al.* 2004). Levodopa-induced changes in striatal gene expression are believed to result form abnormal, intermittent stimulation of supersensitive receptors on DA-denervated striatum because PPE and PDyn mRNA changes can be normalized with long-acting DA agonists that do not induce dyskinesia (Henry *et al.* 1999; Calon *et al.* 2000) and when the same short acting-DA agonists are continuously administered (Morissette *et al.* 1997).

Entacapone is a potent, selective and reversible peripherally acting inhibitor of catechol-O-methyl transferase (COMT), the enzyme that provides the main breakdown pathway for levodopa in the blood after dopa decarboxylase. Inhibition of COMT by entacapone prolongs the half-life of levodopa (Nutt *et al.* 1994; Ruottinen and Rinne 1996) and this is associated with improved clinical efficacy in PD patients (Kaakkola and Wurtman 1993; Nutt *et al.* 1994; Ruottinen and Rinne 1996; Parkinson Study Group 1997; Rinne 1998; Poewe 2002, Stocchi *et al.* 2004; Grandas *et al.* 2007; Deuschl *et al*. 2007; Müller *et al*. 2007) and in experimental models of parkinsonism (Smith *et al*. 2003; 2005; Marin *et al*. 2005, 2006). By extending the half-life of levodopa with entacapone, it is possible to deliver the drug in a way that is less pulsatile, thereby allowing the benefit of levodopa with a reduced risk for motor complications. In detail, the administration of entacapone attenuates and prevents the development of levodopa-induced motor fluctuations (Marin *et al.* 2005) and dyskinesias in 6-OHDA-lesioned rats (Marin *et al*. 2006) and in MPTP-treated monkeys (Smith *et al.* 2003, 2005). However, it is still unknown whether the administration of entacapone modifies the molecular changes induced by levodopa in the basal ganglia nuclei.

The motor response to levodopa is comprised of two components: the long duration response (LDR) and the short duration response (SDR) (Muenter and Tuce 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 the motor fluctuation known as "wearing-off". However, 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). A study in the *novo* patients (the ELLDOPA trial) has shown that the LDR may actually last for several weeks after cessation of levodopa treatment (Parkinson Study Group 2004). An experimental model of the LDR to levodopa has been recently described in 6-OHDA-lesioned rats (Marin *et al.* 2007). An improvement in forelimb akinesia that lasted for at least 2 days after levodopa discontinuation resembling the LDR to levodopa in patients, has been observed (Marin *et al*., 2007). The effect of entacapone on the LDR to levodopa is still unkonwn.

Based in the hypothesis that entacapone may provide a greater bioavailability of levodopa in the brain and thus, a more continuous stimulation of dopaminergic receptors, we investigated if entacapone administration normalizes the molecular alterations induced by levodopa treatment. We tried to define in more detail the mechanism of action by which levodopa plus entacapone

administration reduces motor complications in the 6-OHDA rat model by assessing The effect of entacapone on: (i) the LDR to levodopa, (ii) the postsynaptic pharmacodynamic mechanisms induced by levodopa, and (iii) the levodopa-induced molecular changes in the basal ganglia nuclei. A better definition of putative postsynaptic mechanisms mediating the entacapone effect may beclinical relevant as they may be involved in the development or maintenace of levodopa-induced motor complications and thus, might be avoided with future treatment strategies.

Material and Methods

6-OHDA lesions

Forty-four male Sprague-Dawley rats weighing 220-240 g were housed on a 12-hour light/dark cycle with free access to food and water. Under sodium pentobarbital anesthesia (50 mg/kg, intraperitoneal, i.p.), rats were placed in a stereotactic frame with the incisor bar positioned 4.5 mm below the interaural line. Each animal received a 6-OHDA (Sigma, Spain) injection (8 µg in 4 µl of saline with 0.02% ascorbate over 8 minutes) into the left medial forebrain bundle by means of a Harvard infusion pump. Stereotactic 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 and Watson (1986). Adequate measures were taken to minimize pain or discomfort. All animal experiments were carried out in accordance with the National Institutes of Health guide for care and use of laboratory animals and approved by the local Government.

Protocol of treatments

Animals were distributed in three groups and treated with (1) levodopa methyl ester (25 mg/kg with 6.25 mg/kg benserazide, i.p.) (Sigma, Spain) plus vehicle (n=16) twice a day; (2) levodopa methyl ester (25 mg/kg with 6.25 mg/kg benserazide, i.p.) plus entacapone (30 mg/kg, i.p., n= 18) twice a day, or vehicle (n= 10) for 22 consecutive days.

Experimental design

Three sets of experiments were performed in order to investigate the effect of chronic entacapone administration on: i) levodopa-induced LDR ii) levodopa-induced postsynaptic pharmacodynamic alterations and, iii) levodopa-induced molecular changes in the basal ganglia nuclei.

In the first set, the effect of chronic entacapone on levodopa-induced LDR was evaluated. The effect of entacapone on the use of the parkinsonian limb induced by levodopa was investigated by performing the cylinder test. LDR 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 or levodopa plus entacapone chronic treatment (Marin *et al.* 2007).

In the second set of experiments, we evaluated whether postsynaptic pharmacodynamic mechanisms were involved in the entacapone effect on levodopa-induced motor response. The rotational response to apomorphine (0.5 mg/kg, sc) (Sigma, Spain) was evaluated 3 days after the last treatment day in both, levodopa plus vehicle and levodopa plus entacapone, treated animals.

And finally, to investigate the effects of entacapone on levodopa-induced molecular changes in the basal ganglia nuclei, groups of animals treated following the same protocol than for

the behavioral studies, were sacrificed after 3 days of washout and the in situ hybridization studies were performed.

Rotational Screening

For the measurement of rotational behavior, rats were placed in circular cages and tethered to an automated rotometer. The number of complete (360º) turns made during each 5-minute period was automatically recorded by a computerized system. Rats were allowed 15 minutes to habituate to the rotometer before drug administration. Following a three-week recovery period, rats exhibiting a vigorous rotational response (>100 total turns) to apomorphine (0.05 mg/kg, subcutaneous (sc) were selected for further study. It has been previously demonstrated that rats meeting this criterion have a greater than 95% depletion of striatal dopamine (Papa *et al.* 1994). Apomorphine-induced rotations (0.5 mg/kg, sc) were measured to evaluate the involvement of postsynaptic mechanisms in entacapone effects.

Forelimb akinesia-Cylinder test

To assess forelimb akinesia the cylinder test was used as previously described (Schallert *et al*. 2000; Marin *et al.* 2007). 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 forelimb 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, 3 days after and then weekly during levodopa or levodopa plus entacapone treatment, followed by evaluations at 4h, 2, 5 and 7 days after levodopa or levodopa plus entacapone withdrawal.

Tissue collection

Three days after last levodopa, levodopa plus entacapone or saline administration rats were sacrificed under an overdose of pentobarbital anaesthesia. Brains were quickly removed from the skull and then frozen on dry ice and kept at -80ºC until were cut on a cryostat. Coronal 14 μm thick sections were collected through the striatum onto APTS (3-amino-propyltriethoxysilane) coated slides, and kept at -40ºC until used.

Dopamine transporter (DAT) immunohistochemistry

Striatal sections were thawed and dried at room temperature and fixed with acetone for 10 min at 4ºC. The sections were rinsed in PBS (phosphate buffered saline, 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 4ºC with mouse anti-DAT monoclonal antibody (SantaCruz Biotechnology, Spain) at a dilution 1:500 in PBS. Sections were rinsed twice in PBS, 5 min each, and ImmunoPure Ultra-Sensitive ABC Peroxidase staining kit (Pearce, Spain) was used to carry out the ABC staining method. By so doing, sections were incubated with biotinylated horse anti-mouse Ig-G 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): preprodynorphin, bases 607-654 [NM_019374]; preproenkephalin, bases 513-542 [K02807]; cytochrome oxidase subunit I, bases 644-688 [S79304]; glutamic acid decarboxylase 67 (GAD67), bases 191-235 and 1600-1653 [NM_01700]; dopamine D3 receptors, bases 85-132, 1144-1191 and 1381-1426 [NM_017140]. They were synthesized and HPLIC purified by Amersham Pharmacia Biotech (UK) and by Isogen Bioscience BV (Maarsden, The Netherlands). The oligonucleotides were labeled at their 3'-end by using [^{33}P]dATP (3000 Ci/mmol, New England Nuclear, Boston, MA, USA) and terminal deoxynucleotidyl-transferase (TdT, 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% paraformaldeyde in phosphate-buffered saline (1 x PBS: 2.6 mM KCl, 1.4 mM KH_2PO_4, 136 mM NaCl, 8 mM Na_2HPO_4), washed once in 3 x PBS, twice in 1 x 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 sec in 2mg/ml glycine in 1x PBS. Tissues were finally rinsed in 1x PBS and dehydrated through a graded series of ethanol. For hybridization, radioactively-labeled probes were diluted to a final concentration of 10^7 cpm/ml in a solution containing 50%

formamide, 4x SSC (1x SSC: 150 mM NaCl, 15 mM sodium citrate), 1x Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 10% dextran sulfate, 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 ºC and then washed 4 times (45 min each) in 600 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA at 60 ºC. Hybridized sections were exposed to BIOMAX-MR film (Kodak) for 1 day to five days depending on the probe used at -70ºC 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 unlabeled 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 ºC but they were completely absent from sections washed at 80 ºC. No such decrease was observed in the background levels of the signal (data not shown).

The average densities of the mRNAs in different brain regions were evaluated semi quantitatively 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 analyzed by analysis of variance (ANOVA) followed by Dunnett's t-test for multiple comparisons or paired Student 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 dopamine transporter (DAT)-immunoreactivity in the ipsilateral striatum (Figure 1). No significant differences in the degree of dopaminergic denervation were observed among the groups of rats that received levodopa, levodopa plus entacapone or saline administration (Figure 1).

Effects of chronic administration of levodopa plus entacapone on forelimb akinesia

Prior to 6-OHDA lesion, the proportion of wall contacts performed with the right paw amounted to 55% and 52% of total in levodopa and levodopa plus entacapone-treated animals, respectively. This percentage of wall contacts with the right (parkinsonian) paw was minimal 3 weeks after 6-OHDA lesion in all animals ($p<0.01$) (Figure 2).

During levodopa (25 mg/kg with 6.25 mg/kg benserazide) or levodopa plus entacapone (30 mg/kg) treatment, the limb asymmetry was measured 45 minutes after a test dose of levodopa (6 mg/kg), (Figure 2). Forelimb akinesia was evaluated before and after 6-OHDA lesion, 3 days after and then weekly during levodopa or levodopa plus entacapone treatment, followed by evaluations at 4h, 2, 5 and 7 days after levodopa or levodopa plus entacapone withdrawal. A similar significant improvement in limb use asymmetry was observed in both group of animals chronically treated with levodopa or levodopa plus entacapone. 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. The increased limb activity in the levodopa-treated group was significant up to 48 h after levodopa treatment withdrawal, as previously reported (Marin *et al.* 2007) (Figure 2). In the group treated with levodopa plus entacapone the benefit lasted for 7 days after treatment withdrawal.

Effects of chronic administration of levodopa plus entacapone on apomorphine-induced rotational behavior

No differences were observed in the total number of contralateral rotations induced by apomorphine between both levodopa and levodopa plus entacapone treated groups (Figure 3a). The administration of entacapone did not decrease the maximal peak of rotation induced by apomorphine and the duration of the rotational behavior was not significantly modified (Figure 3b).

Effects of chronic administration of levodopa plus entacapone on molecular markers in basal ganglia

Striatal PPE mRNA expression

As expected, a significant increase in PPE mRNA levels in the lesioned striatum was observed after 6-OHDA lesion in the saline-treated animals compared with the intact striatum ($p<0.01$) (Figure 4). Levodopa treatment maintained the increase in striatal PPE levels ($F_{2,11}$: 3.04)). In the group of animals treated with levodopa plus entacapone, the PPE mRNA levels did not differ from the ones achieved by levodopa treatment (Figure 4). PPE mRNA expression levels were not modified in the intact striatum of any group.

Striatal PDyn mRNA expression

Striatal PDyn mRNA levels were significantly reduced in the 6-OHDA- lesioned striatum ($p<0.01$) in the saline-treated animals compared with the intact striatum, as expected (Figure 5). Levodopa treatment induced a significant increase in PDyn mRNA in the lesioned striatum in comparison with saline-treated rats ($p<0.01$) ($F_{2,13}$: 27.53) and with intact side ($p<0.05$). In the group of animals treated with levodopa plus entacapone, the PDyn mRNA levels did not differ from the ones achieved by levodopa treatment (Figure 5). There were no significant changes in PDyn mRNA expression levels in the intact side in any group.

Striatal dopamine D-3 receptor mRNA expression

Dopamine D-3 receptor mRNA expression was not modified by the 6-OHDA lesion compared with the intact striatum (Figure 6). Rats that received chronic intermittent treatment with levodopa showed a significant increase in DA D-3 mRNA in the lesioned striatum in comparison with the intact side ($p<0.01$) ($F_{2,12}$: 2.83). In the group of animals treated with levodopa plus entacapone, the D-3 mRNA levels did not differ from the ones achieved by levodopa treatment (Figure 6) There were no significant changes in DA D-3 mRNA expression levels in the intact side in any group.

Subthalamic cytochrome oxidase (CO) mRNA expression

Unilateral 6-OHDA lesion induced a significant increase in CO mRNA expression in the ipsilateral subthalamic nucleus ($p<0.05$) (Figure 7). Rats that received chronic intermittent treatment with levodopa showed a significant decrease in CO mRNA in the STN in comparison with saline-treated rats ($p<0.05$) ($F_{2,13}$: 4.28). This effect was noticeable as the levels were normalized in comparison with the intact side in the same group. In the group of animals treated with levodopa plus entacapone, the subthalamic CO mRNA levels did not differ from the ones achieved by levodopa treatment (Figure 7). 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 in GAD_{67} mRNA expression in the ipsilateral pars reticulata of the substantia nigra ($p<0.05$) (Figure 8). Rats that received chronic intermittent treatment with levodopa showed a significant decrease in GAD_{67} mRNA in the ipsilateral SNr in comparison with saline-treated rats ($p<0.05$) ($F_{2,13}$: 3.22). In the group of animals treated with levodopa plus entacapone, the nigral GAD_{67} mRNA levels did not differ from the ones achieved by levodopa treatment (Figure 8). There were no significant changes in GAD_{67} mRNA expression levels in the SNr contralateral to the lesion.

Discussion

Our present results show that the administration of entacapone increased the LDR induced by levodopa since in the 6-OHDA-lesioned rats treated with levodopa plus entacapone the beneficial motoric effect lasted for, at least, 7 days after treatment discontinuation. In the present study, we used a sub-threshold dose of levodopa as a test to evaluate the LDR to levodopa in animals chronically treated with a higher dose of levodopa, as previously described (Marin *et al.* 2007). Using such paradigm, we have observed an improvement in forelimb akinesia that lasted for, at least, 2 days after levodopa discontinuation (Marin *et al.* 2007). The phenomenology of this improvement and its further decay when levodopa is stopped resemble the LDR to levodopa. The potentiation of the LDR to levodopa by entacapone administration agrees with previous observations showing that entacapone attenuates and prevents levodopa-induced motor fluctuations in 6-OHDA-lesioned rats (Marin *et al.* 2005) and PD patients (Parkinson Study Group 1997; Rinne *et al.* 1998; Reichmann *et al.* 2005; Grandas *et al.* 2007). In addition, a beneficial effect of entacapone administration attenuating levodopa or DA agonist-induced dyskinesias in 6-OHDA-lesioned rats (Marin *et al.* 2006) and in MPTP-treated monkeys (Smith *et al.* 2003, 2005, Zubair *et al.* 2007) has been already shown. Because it has been associated with levodopa, a presynaptic mechanism has been considered as one of the mechanisms involved in the LDR (Quattrone *et al*. 1995; Nutt and Holford 1996). However, a LDR can be produced in the novo patients with the dopamine agonist apomorphine (Nutt and Carter 2000) suggesting the involvement of postsynaptic pharmacodynamic mechanisms.

To address the possible postsynaptic mechanisms involved in the entacapone effect on levodopa-induced motor responses, we have measured the rotations induced by apomorphine 3 days after last treatment administration (Juncos *et al.* 1989). The action of apomorphine is mediated through postsynaptic D1 and D2 receptors and is not dependent of presynaptic mechanisms as occurs with levodopa. Juncos *et al.* (1989) found that continuous levodopa administration attenuated the increased rotational behavior response to apomorphine induced after intermittent administration indicating that the postsynaptic mechanisms involved in the motor effects after pulsatile stimulation of DA receptors were attenuated by the continuous administration of levodopa. In the present study, we show that coadministration of entacapone did not modify the rotational response to apomorphine in comparison with the one elicited by levodopa alone. This result suggests that postsynaptic mechanisms might not be involved in the entacapone effects on levodopa-induced motor responses such as LDR

Abundant experimental evidence indicates that discontinous dopaminergic stimulation leads to dysregulation of genes and proteins in the basal ganglia nuclei in PD animal models , that in turn may lead to abnormal neuronal firing patterns that have been associated with dyskinesia in PD (Morissette *et al.* 1997; Vitek and Giroux 2000; Levy *et al*. 2001; Aubert *et al.* 2005; Alonso-Frech *et al.* 2006). Neither, the gene changes nor the dyskinesia associated with a short–acting dopaminergic drug are reported when the same drug is given by continuous infusion (Morissette *et al.* 1997). In detail, the enkephalin precursor PPE mRNA is co-expressed with gammaaminobutyric acid (GABA)-striatal D-2 bearing neurons and levels of PPE mRNA are upregulated after a DA lesion (Gerfen *et al*. 1990; Jolkkonen *et al.* 1995; Morissette *et al*. 1997; Henry *et al*. 1999; Bezard *et al.* 2001; Ravenscroft *et al.* 2004; Marin *et al.* 2007). In 6-OHDA-lesioned rats, repeated administration of levodopa elicits and enhanced behavioral response that is associated with an increased striatal PPE mRNA expression (Ravenscroft *et al.* 2004; Chen *et al.* 2005; Marin *et al.* 2007). However, following repeated administration of antiparkinsonian agents with longer half-life such as bromocriptine, which does not cause behavioral enhancement, PPE mRNA expression is not increased (Henry *et al.* 1999). In MPTP monkeys, intermittent levodopa administration induces dyskinesia in association with persistent or even further upregulation of striatal PPE (Jolkkonen *et al.* 1995; Morissette *et al.* 1997, 1999). In contrast, long-acting dopamine agonists do not induce dyskinesia, and PPE levels are down-regulated (Morissette *et al.* 1999). Moreover, when dyskinesias are induced by intermittent delivery of short-acting DA agonists, PPE levels remain up-regulated whereas when the same agonist is administered continuously, the animals do not experience dyskinesia and PPE levels are down-regulated (Morissette *et al.* 1997). On the other hand, PDyn mRNA that is expressed in the direct pathway is decreased in 6-OHDA lesioned rats (Henry *et al.* 1999; Ravenscroft *et al.* 2004). Intermittent levodopa administration reverses, or further increases, the decrease in striatal PDyn mRNA (Andersson *et al*. 1999; Henry *et al.* 1999; Ravenscroft *et al.* 2004, Marin *et al*. 2007). However, long-acting drugs normalize the PDyn mRNA to pre-lesion levels (Henry *et al.* 1999).

All of the above observations suggest that the motor improvement and the attenuation of motor complications-induced by the CDS are associated with a normalization of levodopa or DA agonist-induced molecular changes in the basal ganglia. From a theoretical point of view, provision of a constant supply of DA or a DA agonist to striatal DA receptors should mimic the state seen during normal tonic firing of dopaminergic receptors, by avoiding fluctuations in DA levels that accompany intermittent levodopa dosing and thus facilitate more normal control of movement (Olanow *et al*. 2006).

A promising therapeutic strategy for PD focused on the potential of providing CDS to ameliorate or prevent levodopa-related motor complications have been developed using

COMT inhibitors such as entacapone (Fenelon *et al.* 2003; Deuschl *et al.* 2007; Grandas *et al*. 2007; Müller *et al.* 2007). Administration of a single dose of levodopa with a COMT inhibitor increases the levodopa plasma area under the curve and its elimination half-life without augmenting peak plasma levels (Nutt *et al.* 1994; Heikkinen *et al*. 2002). According to these pharmacokinetic features, the administration of levodopa with a COMT inhibitor could maintain more stable plasma levels, thus providing an increase in the bioavibility of levodopa and an increase in a longer duration of striatal DA receptor stimulation (Heikkinen *et al.* 2002; Gerlach *et al.* 2004; Paija *et al.* 2005). Until present, it was unknown whether the COMT inhibitor entacapone normalizes levodopa-induced molecular changes.

Our present results show that the coadministration of entacapone with levodopa was not able to normalize levodopa-induced molecular changes in the basal ganglia nuclei. In detail, entacapone administration did not modify the increase in striatal PPE, PDyn or D-3 mRNAs induced by levodopa. No significant differences were observed in CO mRNA in the subthalamic nucleus and in the GAD67 mRNA in the SNr between levodopa alone and levodopa plus entacapone groups. Altogether these data indicates that the potentiation of the LDR to levodopa shown in the present study and the attenuation of levodopa-induced motor fluctuations and dyskinesias by entacapone previously reported (Marin *et al*. 2005, 2006) are not related with a normalization of the molecular changes induced by levodopa in the basal ganglia nuclei. However, these observations do not contradict one to each other. The pharmacokinetics and pharmacodynamics of entacapone need to be taken in account. Microdialysis studies in rats showed that entacapone is able to enhance striatal DA output following administration of levodopa (Kaakola *et al*. 1993; Törnwall *et al.* 1994). The increased DA efflux produced by COMT inhibitors is thought to result from the higher brain availability of levodopa consequent to the reduced peripheral conversion of the amino acid into 3-*O*-methyl-DOPA (3-OMD). It has been shown that central COMT inhibitors in comparison with peripheral inhibitors, such as entacapone, have the additional capacity to further extend the half-life of levodopa being more effective than entacapone in increasing extracellular striatal levels of DA (Napolitano *et al.* 2003) and, thus, in stimulating the DA receptors in a more continuous manner.

Thus, plasma levels of levodopa and consequent DA striatal concentrations achieved by administering levodopa plus benserazide plus entacapone twice daily are probably insufficient to achieve CDS and therefore, incapable to normalize levodopa-induced molecular changes. Indeed, the half-life of entacapone might also be relevant to the lack of modification of levodopa-induced molecular alterations since it has been shown that is shorter than central COMT inhibitors (Forsberg *et al*. 2003). Our findings as well as clinical pharmacokinetics studies (Stocchi *et al.* 2005) indicate that the triple combination of levodopa plus benserazide plus entacapone should be given several times per day (i.e. 4

times) in order to minimize the risks of developing motor complications. This has practical clinical implications in the designs of trials and for routine practice where levodopa plus benserazide plus entacapone is usually administered thrice a day.

Acknowledgements

This work was supported by an unrestricted grant from Novartis-Orion Pharma (Barcelona, Spain). JAO serves as external adviser for Novartis Pharmaceutical (Barcelona, Spain). EA is partially financed by the program: *Ayudas para Contratos de Apoyo a la Investigación en el Sistema Nacional de Salud* from the *Ministerio de Sanidad y Consumo* of the Spanish Government.

References

Abercrombie E.D., Bonatz A.E. and Zigmond M.J. (1990) Effects of L-Dopa on extracellular dopamine in striatum of normal and 6-hydroxydopamine-treated rats. *Brain Res.* **525**, 36-44.

Alonso-Frech F., Zamarbide I., Alegre M., Rodríguez-Oroz M.C., Guridi J., Manrique M., Valencia M., Artieda J. and Obeso J.A. (2006) Slow oscillatory activity and levodopa-induced dyskinesias in Parkinson's disease. *Brain* **129**, 1748-1757.

Andersson M., Hilbertson A. and Cenci M.A. (1999) Striatal fos B expression is causally linked with L-Dopa-induced abnormal involuntary movements and the associated upregulation of striatal prodynorphin mRNA in a rat model of Parkinson's disease. *Neurobiol. Dis*. **6**, 461-474.

Aubert I., Guigoni C., Hâkansson K., Qin L., Dovero S., Barthe N., Bioulac B.H., Gross C.E., Fisone G., Bloch B. and Bezard, E. (2005) Increased D1 dopamine receptor signaling in levodopa-induced dyskinesia. *Ann. Neurol*. **57**, 17-26.

Bédard P.J., DiPaolo T., Falardeu P. and Boucher R. (1986) Chronic treatment with L-Dopa, but not bromocriptine induces dyskinesia in MPTP-parkinsonian momkeys: Correlation with [3H] spiperone binding. *Brain Res.* **379**, 294-299.

Bezard E., Ravenscroft P., Gross C.E., Crossman A.R. and Brotchie J.M. (2001) Upregulation of striatal preproenkephalin gene expression occurs before the appearance of parkinsonian signs in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine monkeys. *Neurobiol. Dis.* **8**, 343-350.

Blanchet P.J., Calon F., Martel J.C., Bédard P.J., Di Paolo T., Walters R.R. and Piercey M.F. (1995) Continuous administration decreases and pulsatile administration increases behavioral sensitivity to a novel dopamine D-2 agonist (U-91356A) in MPTP-exposed monkeys. *J. Pharmacol. Exp. Ther.* **272**, 854-859.

Bychkov E., Ahmed M.R., Dalby K.N. and Gurevich E.V. (2007) Dopamine depletion and subsequent treatment with L-DOPA, but not the long-lived dopamine agonist pergolide, enhances activity of the Akt pathway in the rat striatum. *J. Neurochem.* **102**, 699-711.

Calon F., Hadj Tahar A., Blanchet P.J., Morissete M., Grondin R., Goulet M., Doucet J.P., Robertson G.S., Nestler E., Di Paolo T. and Bédard P.J. (2000) Dopamine-receptor stimulation: biobehavioral and biochemical consequences. *Trends Neurosci.* **23** (Suppl l), S92-S100.

Carta A.R., Pinna A., Cauli O. and Morelli, M. (2002) Differential regulation of GAD67, enkephalin and dynorphin mRNAs by chronic-intermittent L-dopa and A_{2A} receptor blockade plus L-Dopa in dopamine-denervated rats. *Synapse* **44**, 166-174.

Cenci M.A., Lee C.S. and Björklund A. (1998) L-Dopa-induced dyskinesia in the rat is associated with striatal overexpression of prodynorphin-and glutamic acid decarboxylase mRNA. *Eur. J. Neurosci*. **10**, 2694-2706.

Chase T.N., Baronti F., fabbrini G., Heuser I.J., Juncos J.L. and Mouradian M.M. (1989) Rationale for continuous dopaminomimetic therapy of Parkinson's disease. *Neurology* **39**, S7-S10.

Chase T.N., Engber T.M. and Mouradian M.M. (1994**)** Palliative and prophylatic benefits of continuously administered dopaminomimetics in Parkinson's disease. *Neurology* **44**, S15-S18.

Chen L., Togasaki D.M., Langston J.W., DiMonte D.A. and Quik, M. (2005) Enhanced striatal opioid receptor-mediated G-protein activation in L-Dopa-treated monkeys. *Neuroscience* **132**, 409-420.

De la Fuente-Fernández R., Schulzer M., Mak E., Calne D.B. and Stoessl A.J. (2004) Presynaptic mechanisms of motor fluctuations in Parkinson's disease: a probabilistic model. *Brain* **127**, 888-899.

Deuschl G., Vaitkus A., Foc G., Roscher T., Schremmer D., Gordin A., and CAMP Study Group (2007) Efficacy and tolerability of entacapone versus cabergoline in parkinsonian patients suffering from wearing-off. *Mov. Dis.* **11**, 1550-1555.

Fabbrini G., Brotchie J.M., Grandas F., Nomoto M. and Goetz C.G. (2007) Levodopa-induced dyskinesias. *Mov. Dis.* **22**, 1379-1389.

Fenelon G., Gimenez-Roldan S., Montrastruc J.L., Bermejo F., Durif F., Bourdeix I., Pere J.J., Galiano L and Schadrack J. (2003) Efficacy and tolerability of entacapone in patients with Parkinson's disese treated with levodopa plus a dopamine agonist and experiencing wearing-off motor fluctuations. A randomized, double-blind, multicentre study. *J. Neural Transm.* **111**, 1343-1363.

Floresco S.B., West A.R., Ash B., Moore H. and Grace A.A. (2003) Afferent modulation of dopamine neuron firing differentially regulates tonic and phasic dopamine transmission. *Nat. Neurosci.* **6**, 968-973.

Forsberg M., Lehtonen M., Heikkinen M., Savolainen J., Järvinen T., Männistö P.T. (2003) Pharmacokinetics and pharmacodynamics of entacapone and tolcapone after acute and repeated administration: A comparative study in the rat. *J. Pharmacol. Exp. Ther.* **304**, 498-506.

Gardoni F., Picconi B., Ghiglieri V., Polli F., Bagetta V, Bernardi G., Cattabeni F., DiLuca M. and Calabresi P. (2006) A critical interaction between NR2B and MAGUK in L-DOPA-induced dyskinesia. *J. Neurosci.* **26**, 2914-2922.

Gerfen C.R., Engber T.M., Mahan L.C., Susel Z., Chase T.N., Monsma F.J. and Sibley D.R. (1990) D-1 and D-2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science* **250**, 1429-1432.

Gerlach M., van de Buuse M., Blaha C., Bremen D. and Riederer P. (2004) Entacapone increases and prolongs the central effects of L-DOPA in the 6-hydroxydopamine-lesioned rat. *Naunyn Schmiedebergs Arch. Pharmacol.* **370**, 388-394.

Grace A.A. (1991) Phasic versus tonic dopamine release and the modulation of dopamine system responsivity: a hypothesis for the etiology of schizophrenia. *Neuroscience* **41**,1-24.

Grondin R., Goulet M., Di Paolo T. and Bédard P.J. (1996) Cabergoline, a long-acting dopamine D-2-like receptor agonist, produces a sustained antiparkinsonian effect with transient dyskinesias in parkinsonian drug-naive primates. *Brain Res*. **735**, 290-306.

Grandas F., Hernández B. and PRACTICOMT Study Group (2007) Long-term effectiveness and quality of life improvement in entacapone-treated Parkinson's disease patients: the effects of an early therapeutic intervention. *Eur. J. Neurol.* **14**, 282-289.

Heikkinen H., Nutt J.G., LeWitt P.A., Koller W.C. and Gordin A. (2001) The effects of different repeated doses of entacapone on the pharmacokinetics of L-Dopa and the clinical response to L-Dopa in Parkinson's disease. *Clin. Neuropharmacol.* **24**, 150-157.

Heikkinen H., Varhe A, Laine T, Puttonen J, Kela M., Kaakkola S. and Reinikainen K. (2002) Entacapone improves the availability of L-dopa in plasma by decreasing its peripheral metabolism independent of L-dopa/carbidopa dose. *Br J. Clin. Pharmacol.* **54**, 363-371.

Henry B., Crossman A.R. and Brotchie J.M. (1999) Effect of repeated L-Dopa, bromocriptine, or lisuride administration on preproenkephalin-A and preproenkephalin-B mRNA levels in the striatum of the 6-hydroxydopamine-lesioned rat. *Exp. Neurol*. **155**, 204-220.

Herrero M.T., Augood 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.

Jenner P. (2000) The relevance of A_{2A} antagonists to the treatment of Parkinsons's disease: concluding remarks. In: Kase H., Richardson P.J. and Jenner, P. (eds), *Adenosine receptors and Parkinson's disease*. Academic Press, London, pp. 267-273.

Jenner P. (2004) Avoidance of dyskinesia: preclinical evidence for continuous dopaminergic stimulation. *Neurology* **62** (1 Suppl 1), S47-S55.

Jolkkonen J., Granata R., Jenner P. and Marsden, C.D. (1995) Acute and subchronic effects of dopamine agonists on neuropeptide gene expression in the rat striatum. *Neuropeptides* **29**, 109-114.

Juncos J.L**.**, Engber T.M., Raisman R., Susel Z., Thibaut F., Ploska A., Agid Y. and Chase T.N. (1989) Continuous and intermittent levodopa differentially affect basal ganglia function. *Ann. Neurol.* **25**, 473-478.

Kaakkola S. and Wurtman R.J. (1993) Effects of catechol-O-methyltransferase inhibitors and L-3,4-dihydroxyphenylalanine with or without carbidopa on extracellular dopamine in rat striatum. *J. Neurochem.* **60**, 137-144.

Levy R., Dostrovsky J.O., Lang A.E., Sime E., Hutchinson W.D. and Lozano A.M. (2001) Effects of apomorphine on subthalamic nucleus and globus pallidus pars interna in patients with Parkinson's disease. *J. Neurophysiol.* **86**, 249-260.

Maratos **E.C.**, Jackson M.J., Pearce R.K. and Jenner P. (2001) Antiparkinsonian activity and dyskinesia risk of ropinirole and L-Dopa combination therapy in drug naïve MPTP-lesioned common marmosets (Callithrix jacchus). *Mov. Dis*. **16**, 631-641.

Marin C., Aguilar E., Bonastre M., Tolosa E. and Obeso, J.A. (2005) Early administration of entacapone prevents levodopa-induced motor fluctuations in hemiparkinsonian rats. *Exp. Neurol*. **192**, 184-193**.**

Marin C., Aguilar E. and Obeso J.A. (2006) Coadministration of entacapone with levodopa attenuates the severity of dyskinesias in hemiparkinsonian rats. *Mov. Dis.* **21**, 646-653.

Marin C., Aguilar E., Mengod G., Cortés R. and Obeso J.A. (2007) Concomitant short- and long-duration response to levodopa in the 6-OHDA-lesioned rat: a behavioural and molecular study. *Eur. J. Neurosci.* **25**, 259-269.

Miller D.W. and Abercrombie E.D. (1999) Role of high-affinity dopamine uptake and impulse activity in the appearance of extracellular dopamine in striatum after administration of exogenous L-Dopa: studies in intact and 6-hydroxydopamine-treated rats. *J. Neurochem.* **72**, 1516-1522.

Morissette M., Goulet M., Soghomonian J.J., Blanchet P.J., Calon F., Bedard P.J. and DiPaolo, T. (1997) Preproenkephalin mRNA expression in the caudate-putamen of MPTP monkeys after chronic treatment with the D2 agonist U91356A in continuous or intermittent mode of administration: comparison with L-Dopa therapy. *Brain Res. Mol. Brain Res*. **49**, 55-62.

Morissette M., Grondin R., Goulet M., Bedard P.J. and Di Paolo, T. (1999) Differential regulation of striatal preproenkephalin and preprotachykinin mRNA levels in MPTP-lesioned monkeys chronically treated with dopamine D1 or D2 receptor agonists. *J. Neurochem*. **72**, 682-692.

Mouradian M.M., Heuser I.J.E., Baronti F. and Chase T.N. (1990) Modification of central acting dopaminergic mechanisms by continuous levodopa therapy for advanced Parkinson's disease. *Ann. Neurol.* **27**, 18-23.

Muenter M.D. and Tyce G.M. (1971) L-Dopa therapy of Parkinson's disease plasma L-dopa concentration, therapeutic response, and side effects. *Mayo Clinic Proc*. **46**, 231-239.

Müller T., Erdmann C., Muhlack S., Bremen D., Przuntek H. and Woitall D. (2007) Entacapone improves complex movement perdormance in patients with Parkinson's disease. *J. Clin. Neurosci*. **14**, 424-428.

Napolitano A., Bellini G., Borroni E., Zürcher G. and Bonuccelli U. (2003) Effects of peripheral and central catechol-O-methyltransferase inhibition on striatal extracellular levels of dopamine a microdialysis study in freely moving rats. *Parkinsonism. Rel. Dis*. **9**, 145-150.

Nutt J.G. (1990) L-dopa-induced dyskinesia: review, observations and speculations. *Neurology* **40**, 340-345.

Nutt J.G., Woodward W.R., Beckner R.M., Stone C.K., Berggren K., Carter J.H., Gaucher S.T., Hammerstad J.P. and Gordin A. (1994) Effect of peripheral catechol-O-methyltransferase inhibition on the pharmacokinetics and pharmacodynamics of levodopa in parkinsonian patients. *Neurology* **44**, 913-919.

Nutt J.G., Carter J.H. and Woodward W.R. (1995) Long-duration response to levodopa. *Neurology*, **45**, 1613-1616.

Nutt J.G., Holford N.H.G. (1996) The response to levodopa in Parkinson's disease: imposing pharmacological law and order. *Ann. Neurol.* **39**, 561-573.

Nutt J.G. and Carter J.H. (2000) Apomorphine can sustain the long-duration response to L-Dopa in fluctuating PD. *Neurology* **54**, 247-250.

Nyholm D. (2007) The rationale for continuous dopaminergic stimulation in advanced Parkinson's disease. *Parkinsonism Rel. Disord.* **13** (Suppl), S13-S17.

Obeso J.A., Luquin M.R. and Martinez-Lage M. (1986) Intravenous lisuride corrects motor oscillations in Parkinson's disease. *Ann. Neurol.* **19**, 31-35.

Obeso J.A., Grandas F., Herrero M.T. and Horowski R. (1994) The role of pulsatile versus continuous dopamine receptor stimulation for functional recovery in Parkinson's disease. *Eur. J. Neurosci*. **6**, 889-897.

Obeso J.A., Olanow C.W. and Nutt J.G. (2000) Levodopa motor complications in Parkinson's disease. *Trends Neurosci.* **23** (Suppl 10), S2-S7.

Olanow C.W. and Obeso J.A. (2000) Pulsatile stimulation of dopamine receptors and levodopa-induced motor complications in Parkinson's disease: implications for the early use of COMT inhibitors. *Neurology* **55** (suppl 4), 72-81.

Olanow C.W., Obeso J.A. and Stocchi F. (2006) Continuous dopamine-receptor treatment of Parkinson's disease: scientific rationale and clinical implications. *Lancet Neurol.* **5**, 677-687.

Paija O., Laine K., Kultalahti E.R., Leiononen M., Huupponen R., Gordin A. and Reinikainen K. (2005) Entacapone increases levodopa exposure and reduces plasma levodopa variability when used wit Sinemet CR. *Clin. Neuropharmacol.* **28**, 115-119.

Papa S.M., Engber T.M., Kask A.M. and Chase T.N. (1994) Motor fluctuations in levodopa-treated parkinsonian rats: relation to lesion extent and treatment duration. *Brain Res.* **662**, 69-74.

Parkinson Study Group. (1997) Entacapone improves motor fluctuations in levodopa-treated Parkinson's disease patients. *Ann. Neurol*. **4**, 333-341.

Parkinson Study Group. (2004) Levodopa and the progression of Parkinson's disease. *N. Engl. J. Med.***351**, 2498-2508.

Paxinos G., and Watson C. (1986) *The rat brain in stereotaxic coordinates*. Academic Press., New York**.**

Pearce R.K., Banerji T., Jenner P. and Marsden C.D. (1998) De novo administration of ropinirole and bromocriptine induces less dyskinesia than levodopa in MPTP-treated marmoset. *Mov. Dis.* **13**, 234-241.

Pearce R.K. Heikkila M., Lindén I.B. and Jenner P. (2001) L-Dopa induces dyskinesia in normal monkeys: behavioural and pharmacokinetic observation. *Psychopharmacology (Berl)* **156**, 402-409.

Poewe W.H., Deuschl G., Gordin A., Kultalahti E.R. and Leionen M. (2002) Efficacy and safety of entacapone in Parkinson's disease patients with suboptimal levodopa response: a 6 month randomized placebo-controlled double-blind study in Germany and Austria (Celomen study). *Acta Neurol. Scand.* **105**, 245-255.

Quattrone A., Zappia M., Aguglia U., Branca D., Colao R., Montesanti R., Nicoletti G., Palmieri A., Parlato G. and Rizzo, M. (1995) The subacute levodopa test for evaluating long-duration response in Parkinson's disease. *Ann. Neurol*. **38**, 389-395.

Quinn N., Parkes J.D. and Marsden C.D. (1984) Control of on/off phenomenon by continuous intravenous infusion of levodopa. *Neurology* **34**, 1131-1136.

Ravenscroft P., Chalon S., Brotchie J.M. and Crossman A.R. (2004) Ropinirole versus L-Dopa effects on striatal opioid peptide precursors in a rodent model of Parkinson's disease: implications for dyskinesia. *Exp. Neurol*. **185**, 36-46.

Reichmann H., Boas J., Macmahon D., Myllyla V., Hakala A., Reinikainen K. and ComQoL Study Group. (2005) Efficacy of combining levodopa with entacapone on quality of life and activities of daily livingin patients experiencing wearing-off type fluctuations. *Acta Neurol. Scand.* **111**, 21-28.

Rinne U.K., Larsen J.P., Siden A., Worm-Petersen J. and the NOMECOMT Study Group (1998). Entacapone enhances the responses to levodopa in parkinsonian patients with motor fluctuations. *Neurology* **51**, 1309-1314.

Ruottinen H.M. and Rinne U.K. (1996) Entacapone prolongs levodopa response in a one month double blind study in parkinsonian patients with levodopa related fluctuations. *J. Neurol. Neurosurg. Psychtr*. **60**, 36-40.

Schallert T., Fleming S.M., Leasure J.L., Tilerson J.L. and Bland S.T. (2000) CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. *Neuropharmacology* **39**, 777-787.

Smith L.A., Jackson M.J., Hansard M.J., Maratos E. and Jenner P. (2003) Effect of pulsatile administration of levodopa on dyskinesia induction in drug-naive MPTP-treated common marmosets: effect of dose, frequency of administration, and brain exposure. *Mov. Dis.* **18**, 487-495.

Smith L.A., Jackson M.J., Al-Barghouthy G., Rose S., Kuoppamaki M., Olanow W. and Jenner P. (2005) Multiple small doses of levodopa plus entacapone produce continuous dopaminergic stimulation and reduce dyskinesias inductionin MPTP-treated drug-naïve primates. *Mov Dis.* **20**, 306-314.

Stocchi F. and Olanow C.W. (2004) Continuous dopaminergic stimulation in early and advanced Parkinson's disease. *Neurology* **62** (Suppl 1), S56-S63.

Stocchi F., Barbato L., Nordera G, Bolner A. and Caraceni T. (2004) Entacapone improves the pharmacokinetic and therapeutic response of controlled release levodopa/carbidopa in Parkinson's patients. *J. Neural Transm.* **111**, 173-180.

Stocchi F., Vaca L, Ruggieri S, and Olanow C.W. (2005) Intermittent vs continuous levodopa administration in patients with advanced Parkinson's disease: a clinical and pharmacokinetic study. *Arch. Neurol.* **62**, 905-910.

Törnwall M., Kaakkola S., Tuomainen P., Kask A. and Männistö P.T. (1994) Comparison of two new inhibitors of catechol-O-methylation: a microdialysis study in rats. *Br. J. Pharmacol.* **112**, 13-18.

Venton B.J., Zhang H., Garris P.A., Phillips P.E., Sulzer D. and Wigt¡htmann R.M. (2003) Real-time decoding of dopamine concentration changes in the caudate-putamen during tonic and phasic firing. *J. Neurochem.* **87**, 1284-1295.

Vitek J.L. and Giroux, M. (2000) Physiology of hypokinetic and hyperkinetic movement disorders: model for dyskinesia. *Ann. Neurol.* **47**, S131-S140.

Winkler C., Kirik D., Björklund A. and Cenci M.A. (2002) L-Dopa-induced dyskinesia in the striatal 6-hydroxydopamine model of Parkinson's disease: relation to motor and cellular parameters of nigrostriatal function. *Neurobiol. Dis*. **10**, 165-186.

Zappia M., Colao R., Montesanti R., Rizzo M., Aguglia U., Gambardella A., Oliveiri R.L. and Quattrone A. (1997) Long-duration response to levodopa influences the pharmacodynamics of short-duration response in Parkinson's disease. *Ann. Neurol.* **42**, 245-248.

Zubair M., Jackson M.J., Tayarini-Binazir K., Stockwell K.A., Smith L.A., Rose S., Olanow W. and Jenner P. (2007) The administration of entacapone prevents levodopa-induced dyskinesia when added to dopamine agonist therapy in MPTP-treated primates. *Exp. Neurol.* **208**, 177-184.

Legends

Figure 1. Representative DAT immunohistochemistry from 14 □m coronal sections of the rostral striatum. Sections are from rats receiving 6-OHDA injection in the left forebrain bundle, and then treated for 22 days with (A) saline (n=10), (B) levodopa methyl ester (25 mg/kg with 6.25 mg/kg benserazide, i.p.) plus vehicle (n=16) twice a day, and (C) levodopa methyl ester (25 mg/kg with 6.25 mg/kg benserazide, i.p.) plus entacapone (30 mg/kg, i.p., n= 18) twice a day. Note the significant absence of DAT immunoreactivity in the lesioned side regardless of the drug treatment.

Figure 2. Long duration response to levodopa: Forelimb akinesia evaluated 45 minutes after a levodopa dose-test (6 mg/kg, i.p.) in unilateral 6-OHDA-lesioned rats chronically treated for 22 days with levodopa methyl ester (25 mg/kg with 6.25 mg/kg benserazide, i.p.) plus vehicle twice a day, and levodopa methyl ester (25 mg/kg with 6.25 mg/kg benserazide, i.p.) plus entacapone (30 mg/kg, i.p., twice a day. Values are expressed as mean ± SEM. ##p<0.01 vs before 6-OHDA; *p<0.05, **p<0.01 vs 22 days after 6-OHDA.

Figure 3. Effect of entacapone on the postsynaptic mechanisms evaluated by means of apomorphine-induced rotational behaviour. The coadministration of entacapone (30 mg/kg, i.p., twice a day) did not modify the effect of levodopa on apormorphine-induced rotations; a) Total number of rotations; b) time- course curve. Values are expressed as mean ± SEM

Figure 4. Striatal PPE mRNA expression in 6-OHDA-lesioned rats chronically treated treated for 22 days with: levodopa methyl ester (25 mg/kg with 6.25 mg/kg benserazide, i.p.) plus vehicle twice a day; levodopa methyl ester (25 mg/kg with 6.25 mg/kg benserazide, i.p.) plus entacapone (30 mg/kg, i.p.) twice a day or saline. Upper: Values are expressed as mean ± SEM. **p<0.01 vs the corresponding intact side. Bottom: Representative film autoradiograms of coronal brain sections (14 μm) showing striatal PPE mRNA labelling in all treatment groups.

Figure 5. Striatal PDyn mRNA expression in 6-OHDA-lesioned rats chronically treated for 22 days with: levodopa methyl ester (25 mg/kg with 6.25 mg/kg benserazide, i.p.) plus vehicle twice a day; levodopa methyl ester (25 mg/kg with 6.25 mg/kg benserazide, i.p.) plus entacapone (30 mg/kg, i.p.,) twice a day or saline. Upper: Values are expressed as mean ± SEM. *p<0.05, **p<0.01 vs the corresponding intact side; ++p<0.01 vs saline-treated animals. Bottom: Representative film autoradiograms of coronal brain sections (14 μm) showing striatal PDyn mRNA labelling in all treatment groups.

Figure 6. Striatal dopamine D-3 mRNA expression in 6-OHDA-lesioned rats chronically treated for 22 days with: levodopa methyl ester (25 mg/kg with 6.25 mg/kg benserazide, i.p.) plus vehicle twice a day; levodopa methyl ester (25 mg/kg with 6.25 mg/kg benserazide, i.p.) plus entacapone (30 mg/kg, i.p.,) twice a day or saline. Upper: Values are expressed as mean ± SEM. **p<0.01 vs the corresponding intact side. Bottom: Representative film autoradiograms of coronal brain sections (14 µm) showing striatal D-3 mRNA labelling in all treatment groups.

Figure 7. Subthalamic CO mRNA expression in 6-OHDA-lesioned rats chronically treated for 22 days with: levodopa methyl ester (25 mg/kg with 6.25 mg/kg benserazide, i.p.) plus vehicle twice a day; levodopa methyl ester (25 mg/kg with 6.25 mg/kg benserazide, i.p.) plus entacapone (30 mg/kg, i.p.,) twice a day or saline. Upper: Values are expressed as mean ± SEM. *p<0.05 vs the corresponding intact side; #p<0.05 vs saline-treated animals. Bottom: Representative film autoradiograms of coronal brain sections (14 µm) showing subthalamic CO mRNA labelling in all treatment groups.

Figure 8. GAD_{67} mRNA expression in the *pars reticulata* of the substantia nigra in 6-OHDA-lesioned rats chronically treated for 22 days with: levodopa methyl ester (25 mg/kg with 6.25 mg/kg benserazide, i.p.) plus vehicle twice a day; levodopa methyl ester (25 mg/kg with 6.25 mg/kg benserazide, i.p.) plus entacapone (30 mg/kg, i.p.) twice a day or saline. Upper: Values are expressed as mean ± SEM. *p<0.05 vs the corresponding intact side; #p<0.05 vs saline-treated animals. Bottom: Representative film autoradiograms of coronal brain sections (14 µm) showing nigral GAD_{67} mRNA labelling in all treatment groups.

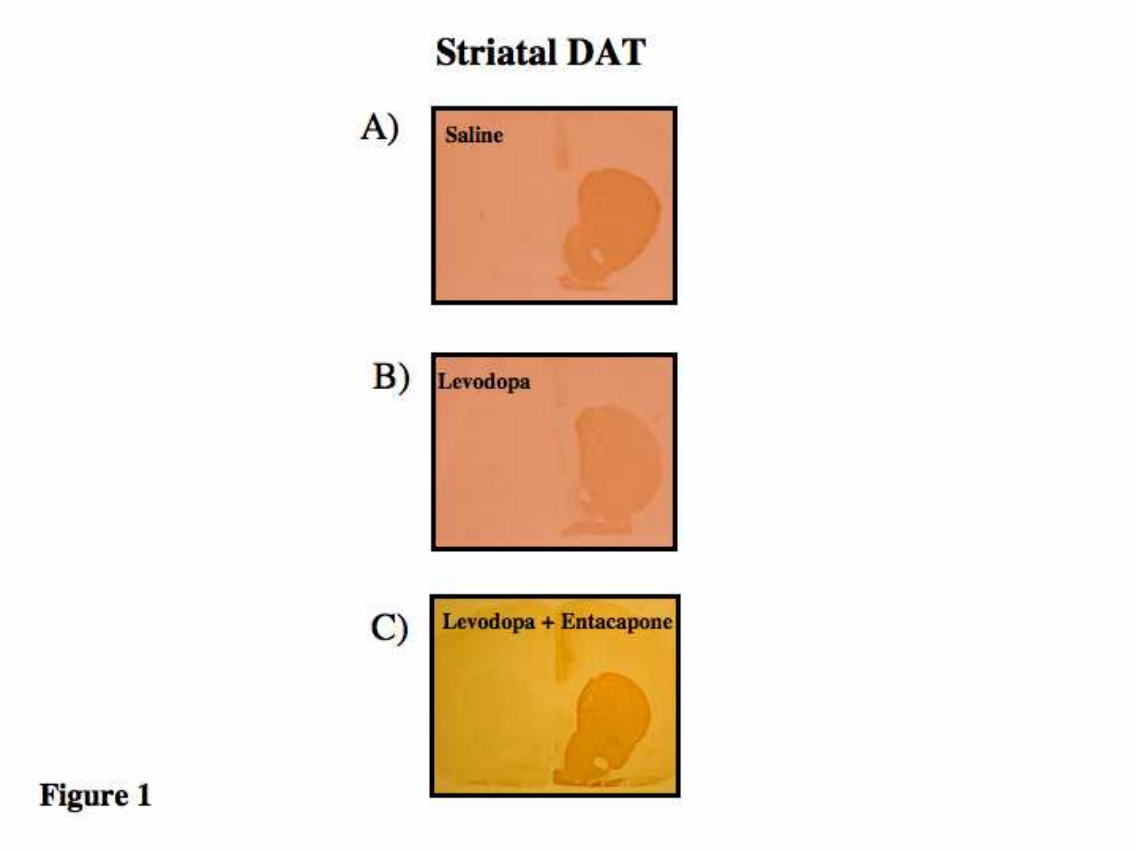

Figure 1

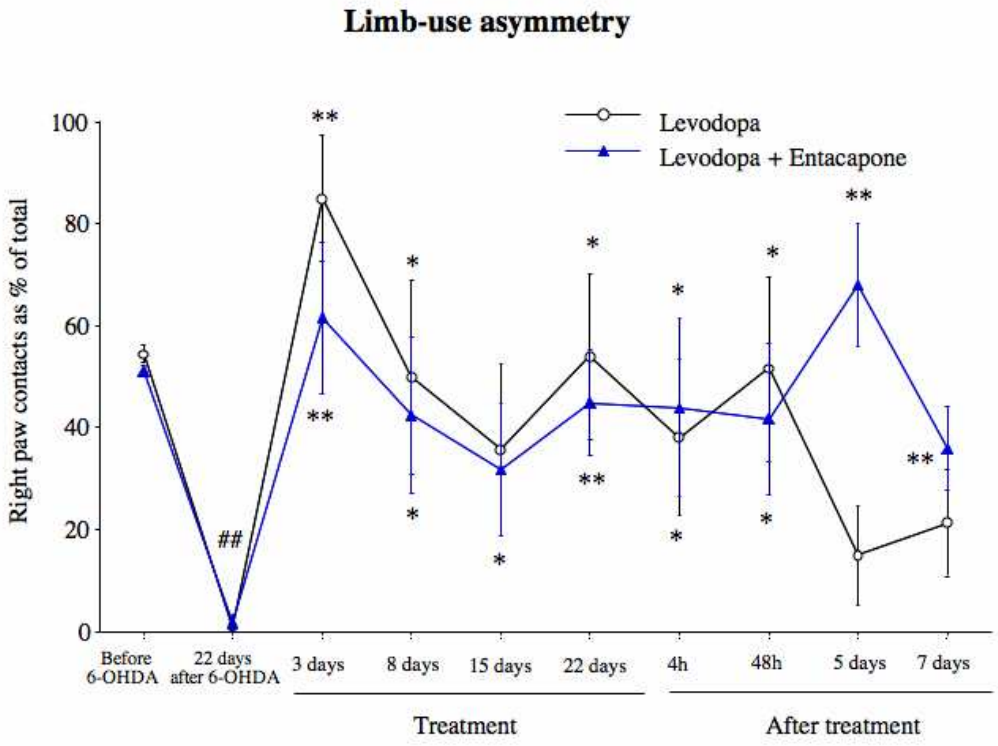

Figure 2

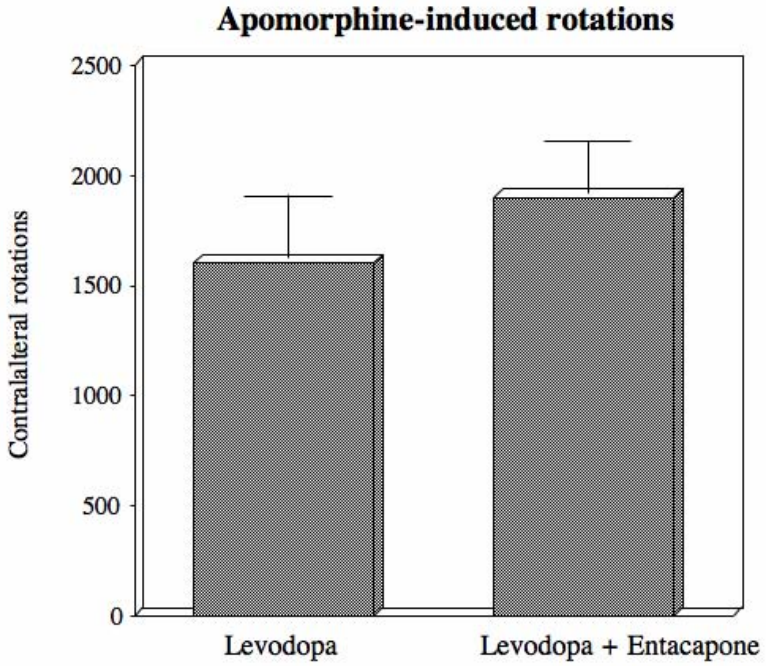

Figure 3a

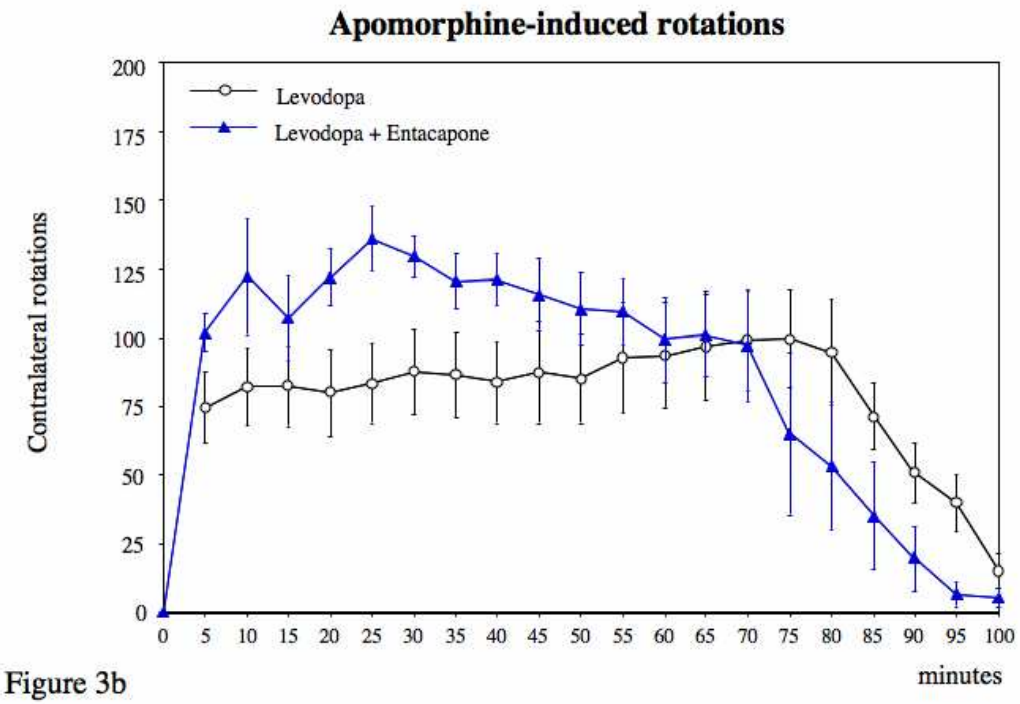

Figure 3b

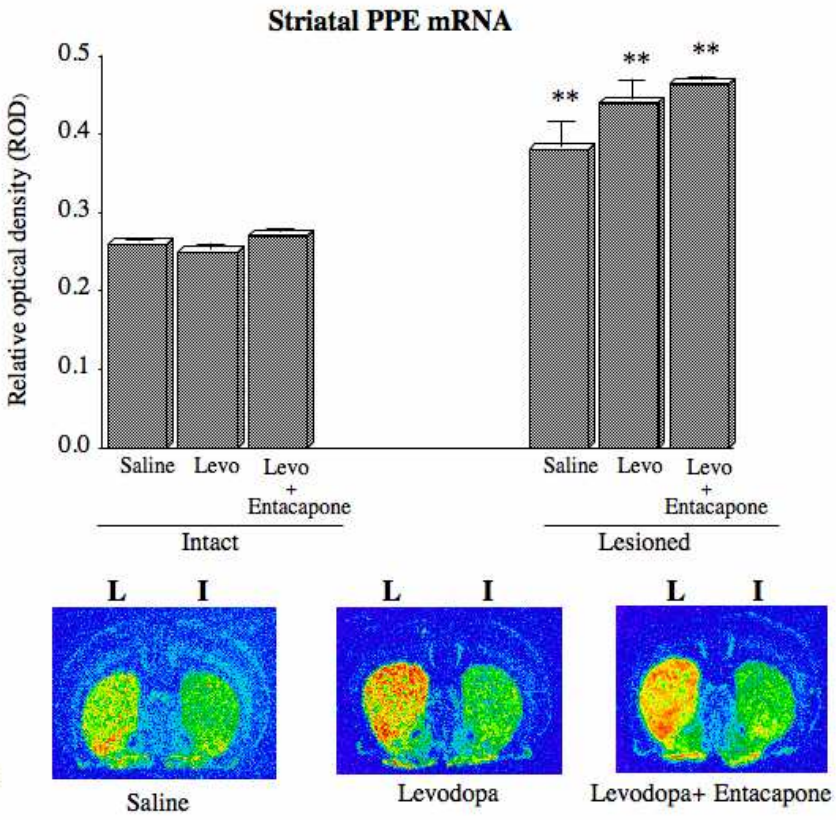

Figure 4

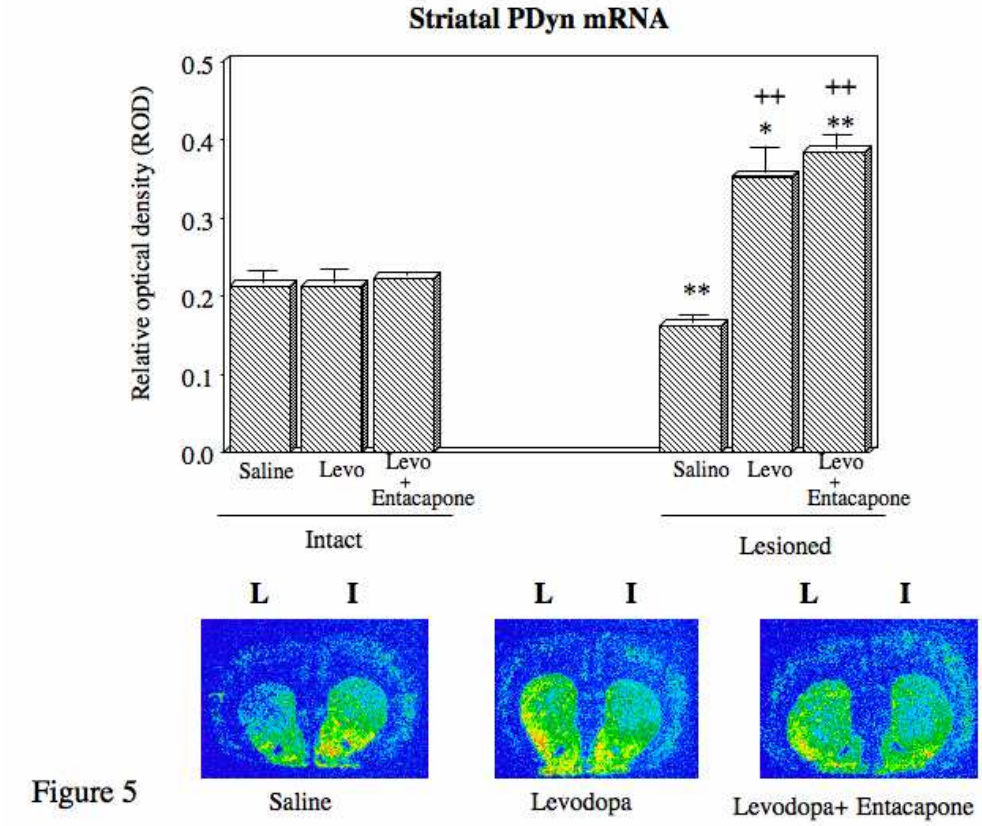

Figure 5

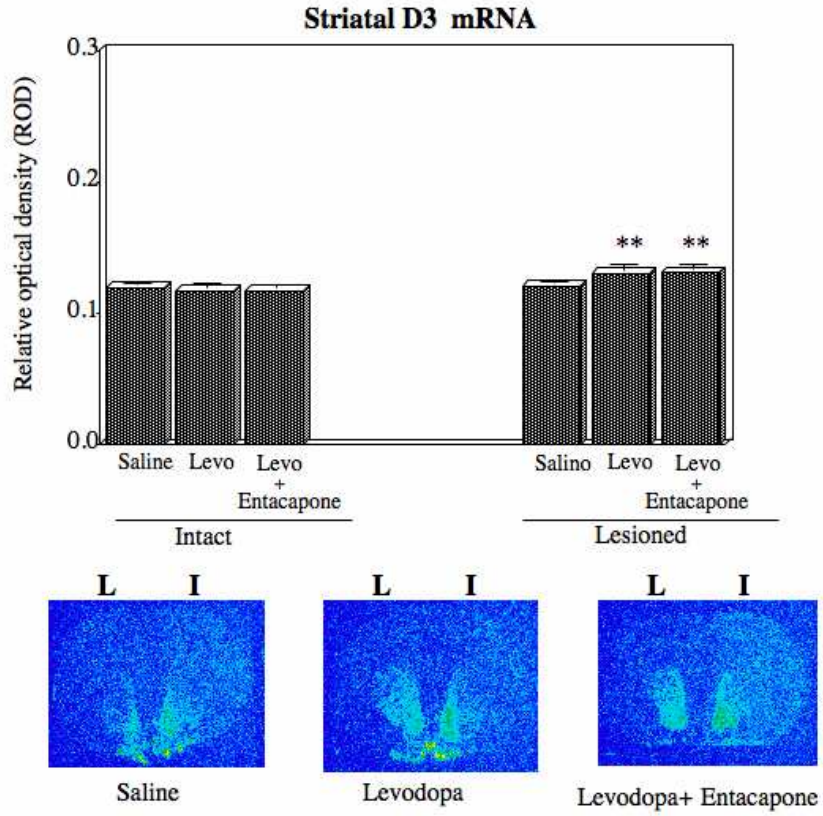

Figure 6

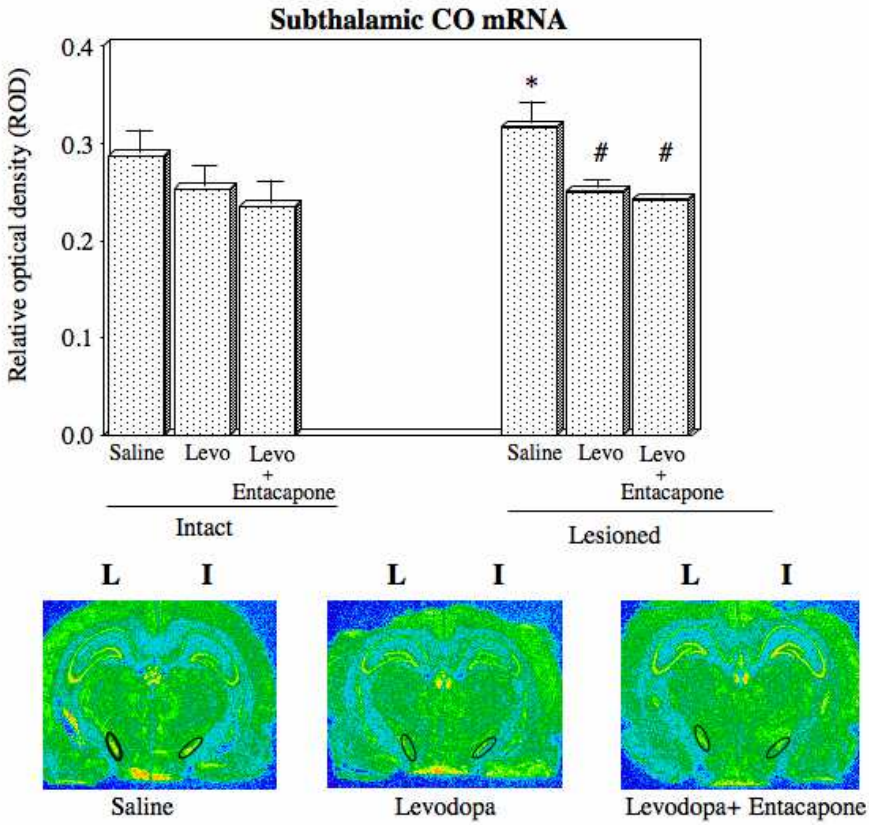

Figure 7

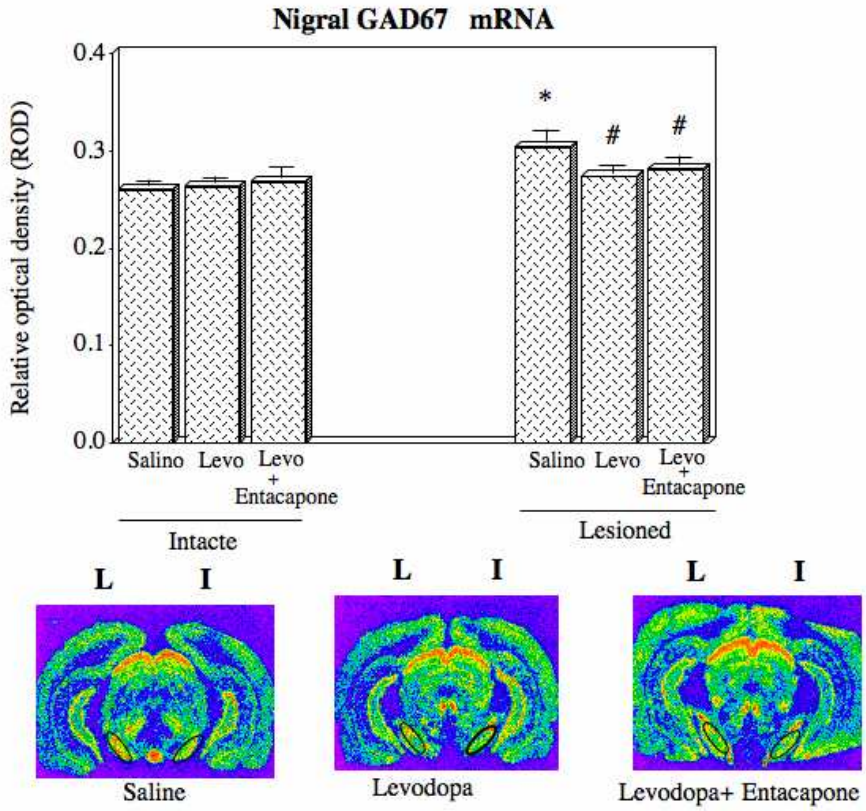

Figure 8