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Sustained improvement of motor function in hemiparkinsonian rats chronically treated with low doses of caffeine or trihexyphenidyl

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Abstract

The effects of chronic oral treatment with low doses of caffeine (1–3 mg/kg) and trihexyphenidyl (0.1–0.2 mg/kg) were tested on hemiparkinsonian rats, which received the following treatments in a counterbalanced order: vehicle, caffeine, trihexyphenidyl, and caffeine plus trihexyphenidyl. Three preclinical models were used: the stepping test, the cylinder test, and the staircase test. Compared to pre-lesion values, the forepaw contralateral to the dopamine-denervated side showed impaired stepping, fewer wall contacts in the cylinder test, and fewer pellets retrieved in the staircase test. In the stepping test both doses of caffeine produced a complete recovery of motor function (100%), whereas the effect of trihexyphenidyl was less intense (77–80%). In this same test the maximal effect of drugs did not develop tolerance during 2–3 weeks, and was completely reversible after drug cessation. In the cylinder test only the wall contacts performed simultaneously with both forepaws were significantly increased by caffeine (3 mg/kg) and trihexyphenidyl (0.2 mg/kg), and this effect was also reversible. In the staircase test none of the treatments improved food pellet retrieval with the contralateral forepaw. Altogether, these results show that chronic treatment with caffeine, at doses similar to daily human consumption, produces a sustained improvement in the use of the contralateral forelimb in unilaterally 6-hydroxydopamine denervated rats, without the development of tolerance. Although the combined administration of caffeine plus trihexyphenidyl showed no synergism in these models, the results suggest that low doses of caffeine (1–3 mg/kg/day) could be of therapeutic value for the reversal of motor symptoms in parkinsonian patients.

Keywords: Parkinson's disease; Adenosine A_{2A} antagonist; Anticholinergic; Trihexyphenidyl; Tolerance; Stepping test; Cylinder test; Staircase test

1. Introduction

The potential of caffeine as a therapeutic adjuvant for the treatment of Parkinson's disease has been recognized for a long time (Fisone et al., 2004). This idea has been supported mainly by preclinical studies showing that caffeine can induce contralateral rotation in rats bearing unilateral 6-hydroxydopamine (6-OHDA) lesions of the dopaminergic nigrostriatal pathway (Casas et al., 1999; Garrett and Holtzman, 1996; Herrera-Marschitz et al., 1988; Watanabe et al., 1981). However, the circling behaviour model, although widely accepted as a useful and inexpensive method for the screening of novel antiparkinsonian compounds (Kaakola and Teravainen, 1990), has several limitations that do not allow verifying whether these drugs can indeed reverse specific motor deficits that occur following striatal dopamine denervation, such as akinesia, postural instability, and loss of fine motor control (Lindner et al., 1996; Lundblad et al., 2002). It is possible that the scarcity of studies aimed at assessing the properties of caffeine in animal models that reproduce the motor deficits of

Abbreviations: 6-OHDA, 6-hydroxydopamine; BF, both forelimbs; CAFF, caffeine; CF, contralateral forelimb; Cpu, caudate-putamen; HD, high dose; IF, ipsilateral forelimb; LD, low dose; POST-B, post-lesion baseline; TH, tyrosine hydroxylase; THP, trihexyphenidyl; VEH, vehicle.

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human parkinsonism with more fidelity has precluded further evaluation of caffeine in placebo-controlled clinical trials as an alternative for the treatment of Parkinson's disease.

Based on the above considerations, we propose that caffeine deserves further evaluation in animal models that, in contrast to the drug-induced rotation, produce motor disabilities more akin to those observed in Parkinson's disease, and that allow a quantitative measurement of the motor improvement induced by drugs (Lindner et al., 1996; Lundblad et al., 2002). Assuming that caffeine is effective at reversing some of the motor deficits in preclinical models of parkinsonism, the second concern is to determine whether this effect is maintained during chronic dosage. This is important since there is evidence that rodents develop tolerance to the rotational response induced by caffeine during chronic administration (Casas et al., 1999). Interestingly, the tolerance produced by high doses of caffeine can be prevented if it is co-administered with the muscarinic antagonist scopolamine (Casas et al., 1999). Moreover, studies performed in pharmacological models of parkinsonism have shown that the anticholinergic trihexyphenidyl enhances the antiakinetic actions of low doses of caffeine (Moo-Puc et al., 2003, 2004). These results suggest that the anticholinergics currently used for the treatment of Parkinson's disease (Fahn et al., 1990) could be combined with caffeine to inhibit the development of tolerance and, perhaps, to increase its antiparkinsonian effects.

Based on the above points, the goal of the present study was to answer the following questions: (1) Are doses of caffeine within the range of habitual human consumption able to reverse some of the motor deficits produced by the loss of striatal dopamine denervation?, (2) Are low doses of caffeine equally effective at improving the postural balance and the fine motor control in parkinsonian animals?, (3) Will the antiparkinsonian effect of low doses of caffeine be sustained during chronic administration?, and (4) Is the muscarinic antagonist trihexyphenidyl able to prevent the tolerance or to enhance the antiparkinsonian actions of low doses of caffeine?

To answer these questions, three clinically relevant animal models that better reproduce some of the motor disabilities of Parkinson's disease were used. The first is the adjusting steps test, which is a model of postural balance that assesses the capacity to regain postural stability and adjust the centre of gravity when a rapid lateral movement is imposed on the animal (Schallert and Tillerson, 2000). The second is the forelimb use asymmetry (cylinder) test that allows the measurement of forelimb placing and shifting during vertical exploratory activity (Schallert and Tillerson, 2000). The third scheme is the paw-reaching staircase test, which evaluates a complex sensorimotor behaviour that might be considered analogous to human reaching and grasping (Montoya et al., 1991). In all these tests the unilateral 6-OHDA lesion of the nigrostriatal dopamine pathway impairs the performance of the contralateral but not the ipsilateral forepaw (Barnéoud et al., 1995; Chang et al., 1999; Lindner et al., 1996; Olsson et al., 1995; Schallert and Tillerson, 2000; Whishaw et al., 1997).

The doses of caffeine (1 and 3 mg/kg) evaluated in these behavioural models were selected on the basis of the following criteria: (a) they cause a significant enhancement of metabolic activity in the basal ganglia of the rat (Nehlig, 1999; Nehlig and Boyet, 2000), (b) they produce a substantial occupancy (>32%) of the striatal adenosine A_{2A} receptors (El Yacoubi et al., 2001), the blockade of which has been associated with antiparkinsonian effects (Ferré et al., 2001; Fisone et al., 2004; Mally and Stone, 1998; Schwarzschild et al., 2002), and (c) they cause a significant inhibition (20–30%) of haloperidol-induced catalepsy in the rat (Moo-Puc et al., 2003). The doses of THP tested were similar to those that enhanced the anticataleptic actions of 1 mg/kg caffeine in rats (Moo-Puc et al., 2003). Part of these results has been presented in abstract form (Bata-García et al., 2005).

2. Methods

2.1. Animals

Experiments were conducted on 13 male Wistar rats bred in our facilities. The initial average weight was 276±6 g (range: 252–310 g), and increased up to 351±5 g (range: 330–400 g) at the end of the experimental period. Animals were individually housed in acrylic cages at constant room temperature (23±1 °C) and maintained on a 12:12 h light/dark cycle (lights on at 07:00) throughout. Food and water were available ad libitum. All efforts were made to minimize animal discomfort according to the recommendations of the Guide for the Care and Use of Laboratory Animals (National Research Council, USA, 1996). This study was approved by the Institutional Bioethics Committee of the CIR-UADY.

2.2. Drugs

Desipramine HCl (Sigma, USA), 6-hydroxydopamine HBr (6-OHDA) (Sigma), trihexyphenidyl HCl (THP) (Sigma), apomorphine HCl (ICN Pharmaceuticals, CA, USA), and caffeine (anhydrous, Fermont-Productos Químicos Monterrey, México) solutions were freshly prepared and protected from light. Desipramine (25 mg/mL) was solubilized in a mixture of distilled water plus dimethylsulphoxide (12.5%). 6-OHDA (3 mg/mL) and apomorphine (0.25 mg/mL) were dissolved in distilled water with ascorbic acid (0.5 mg/mL).

2.3. Lesion

Following a dose of desipramine (25 mg/kg, i.p.) to protect central noradrenergic neurons, rats were anaesthetized with sodium pentobarbital (45 mg/kg, i.p.) and placed in a stereotaxic frame (Stöelting) with the incisor bar set 3.3 mm below the interaural line. A single dose of 6-OHDA (10.5 μg/3.5 μL) was manually injected in small steps (≈0.2 μL/min) into the right substantia nigra pars compacta (SNc), at the coordinates: AP, −5.3 mm from bregma; *L*, −1.8 mm from the midline, and *V*, −7.6 mm from the dura surface (Paxinos and Watson, 1986). Infusion was made through a 30-gauge needle connected with a polyethylene catheter (PE10) to a 10 μL microsyringe (Hamilton™). Upon completion of the injection, the needle was left in place for an additional minute before withdrawal.

In order to select the successfully denervated animals, all rats were tested for apomorphine-induced rotation, starting 14 days after the lesion. The rats were placed in hemispherical bowls (41 cm diameter), secured to a harness and connected with a steel wire to an automated rotometer (Heredia-López et al., 2002). Then a dose of apomorphine (0.25 mg/kg, s.c.) was applied and the rotational behaviour counted during 90 min. The apomorphine-induced rotation test was repeated two more times at weekly intervals. Only rats performing a minimum of 300 complete turns (360°) contralateral to the lesioned side during the last 90-minute test were used in the experiments.

2.4. Treatments

THP and caffeine were administered orally. However, rats refused to ingest THP solubilized in distilled water, perhaps because of its unpleasant flavour. For this reason, several concentrations of the sweetener aspartame (aspartylphenylalanine methylester, Canderel®) were tested, and it was found that a 67 mg/mL solution increased THP acceptance to 100%. Therefore, this solution was used as vehicle for chronic dosage of THP and caffeine. It is unlikely that this dose of aspartame influenced the behavioural tests since doses between 200 and 1000 mg/kg do not modify the rates of brain monoamine synthesis and turnover (Fernstrom et al., 1983; Perego et al., 1988; Torii et al., 1986a) nor the diurnal patterns of spontaneous motor activity (Torii et al., 1986b).

Two dose levels of caffeine and THP were tested in separate groups of rats. In the first series of experiments (high dose group: HD), the tested doses of caffeine (3 mg/kg) and THP (0.2 mg/kg) were slightly higher than those displaying maximal synergism in acute pharmacological models of parkinsonism (Moo-Puc et al., 2003, 2004). It was reasoned that greater doses should compensate for the expected loss by hepatic metabolism after oral administration (Carrillo and Benitez, 2000). Besides, since no synergism between caffeine and THP was found in the former experiments, it was assumed that this was a consequence of a saturating effect. To evaluate this possibility, in a second series of experiments (low dose group: LD), the doses of caffeine (1 mg/kg) and THP (0.1 mg/kg) were the same that produced synergism in other preclinical models (Moo-Puc et al., 2003, 2004).

Within each group, every rat received the four treatments in a counterbalanced order: (A) vehicle (VEH, 1 mL/kg), (B) caffeine (CAFF, 1 or 3 mg/kg), (C) trihexyphenidyl (THP, 0.1 or 0.2 mg/kg), and (D) a mixture of CAFF plus THP, at the corresponding same two doses. Animals of the LD group received treatments in the following sequences: ABCD, CBDA, DBAC, ADBC, DCBA, BADC, ACDB; in the HD group the sequences were: DACB, BADC, BDCA, BCAD, ACDB, ABCD. Each treatment was administered daily for 2 weeks (HD group) or 3 weeks (LD group), as a single oral dose in the morning by means of a steel cannula attached to an insulin syringe. Usually the animals freely ingested the sweetened solution, making it unnecessary to introduce the cannula into the oesophagus. Treatments were separated by a washout period (2 and 3 weeks for groups HD and LD, respectively) in which the animals did not receive any treatment.

2.5. Behavioural testing

Three behavioural tests were used to assess the ability of caffeine and THP to restore the contralateral motor performance after the unilateral 6-OHDA lesion of the mesencephalic dopamine system. All trials were performed between 10:00 h and 18:00 h. The staircase test and the forepaw adjusting steps test were applied on the same days, 45 and 60 min after the oral drug administration. The forelimb use asymmetry test was applied at weekly intervals, 60 min after drug administration.

Fig. 1A shows the sequence of behavioural testing, pre-lesion and post-lesion (baseline), before treatments. Similarly, Fig. 1B depicts the behavioural tests that were applied to assess

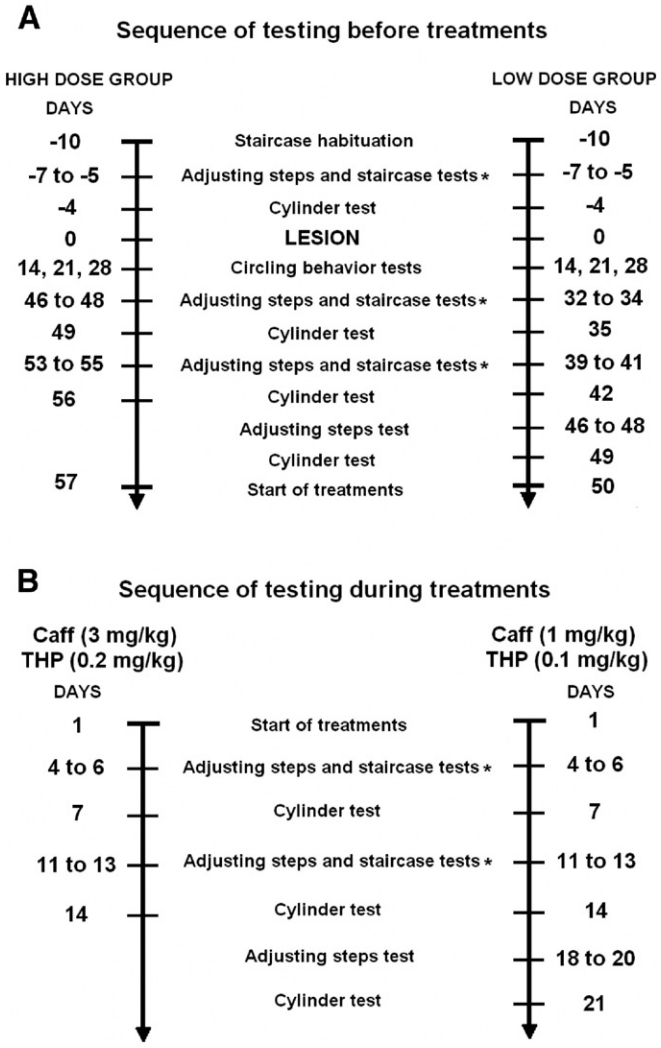

Fig. 1. Sequence of behavioural testing in the high and low dose groups. (A) Sequence of behavioural testing before and after 6-OHDA lesion (baseline). The adjusting steps test and the forelimb use asymmetry test served to evaluate the postural balance, while the staircase test was an index of skilled forepaw reaching. (B) Sequence of behavioural testing during treatments. The staircase test and the forepaw adjusting steps test were sequentially applied, 45 and 60 min after the daily oral dose of drugs, respectively, whereas the forelimb use asymmetry test was executed on different days, 60 min after the treatments. The staircase test was applied only to rats in the high dose group (*). During the drug washout period all tests were applied at the same time of the corresponding days.

motor performance during treatments. The same tests were applied in equivalent days during the washout period.

2.6. Adjusting steps test

The experimenter placed one hand along one side of the rat's body and gently pushed the animal laterally along a smooth stainless steel surface for one meter, at a speed of about 20 cm/s (Lindner et al., 1996). This manoeuvre was performed by first displacing the animal to the left and then to the right. For each animal, the trials were repeated twice (HD group, 6 rats) or four times (LD group, 7 rats) in each direction, and videotaped for later analysis. The sequence of stepping tests executed before and after the 6-OHDA lesions is shown in Fig. 1A. Within the week previous to the surgery, the numbers of adjusting steps performed with the ipsilateral and contralateral forepaws were recorded in three consecutive days. The same procedure was carried out between 4 and 8 weeks after the lesion. For each rat, the pre-lesion and post-lesion baseline values were calculated averaging the adjusting steps performed by each forepaw in the number of daily trials. The single values calculated for each rat were then used to obtain the group average for each day.

2.7. Forelimb use asymmetry (cylinder) test

Rats were placed in a transparent acrylic cylinder and their activity was videotaped for 5 min. The cylinder height (30 cm) was enough to prevent the animal from reaching the top edge by rearing, and its diameter (20 cm) allowed the rat to turn around comfortably while not rearing. A mirror was placed behind the cylinder at an appropriate angle to permit the recording of forelimb movements whenever the animal was turning away from the camera.

The number of wall contacts performed with the forelimbs was counted since it represents a more sensitive index of striatal dopamine depletion than landing movements (Schallert and Tillerson, 2000). Wall contacts were classified as contralateral forelimb (CF), ipsilateral forelimb (IF) or both forelimbs (BF). They were scored as described by Schallert and Tillerson (2000): (a) during a rearing, the first limb used for initial contact on the wall was scored as independent (CF or IF); (b) every lateral movement performed by a single limb contacting the wall was scored as independent (CF or IF); (c) when a limb was already making contact with the wall, the delayed placement of the other (>0.4 s) was scored as simultaneous (BF); (d) if the rat explored the wall laterally, every alternating movement involving both forelimbs was scored as simultaneous (BF); (e) when one limb remained in contact, but stationary, while the other made small adjusting steps, this was scored as one simultaneous contact (BF); (f) when both forelimbs were removed from the wall, any further contact was scored as independent or simultaneous, as described previously. A minimum of 20 wall contacts per trial should occur in order to be considered valid. To stimulate rats that touched the wall less than 20 times, the following procedures were used, in the order listed: (a) covering half of the cylinder top with a sheet of paper, (b) taking the rat out of the cylinder for about 30 s and putting it back in, and (c) turning the lights of the room on and off several times (Lundblad et al., 2002). The percent uses of independent (%CF or %IF) or simultaneous (%BF) forelimbs, relative to the total number of contacts (CF + IF + BF) exhibited by each animal, were calculated.

The forelimb use asymmetry test was performed only at weekly intervals given that exploratory behaviour of rats showed habituation upon daily exposure to the cylinder. The calculated percentage for the test applied on the 4th day before the 6-OHDA lesion, served as the pre-lesion baseline. This test was also carried out on several days after the lesion (Fig. 1A), and the percentage calculated on the last day was taken as the post-lesion baseline.

2.8. Paw-reaching staircase test

A 6-step double staircase apparatus made of Plexiglas® was used, and its shape and dimensions were similar to the device described by Montoya et al. (1991). Food pellets (45±2 mg) were made of chocolate flavoured cereal (Nestlé Crunch®), containing a mixture of rice, corn, wheat, and oats. To enhance food seeking during trials, the standard rat chow was reduced during the preceding 7 days to cause an animal weight loss of about 15%. On the day of the experiment each well of the staircase steps was filled with two food pellets, making a total of 12 pellets per staircase (right and left). The rat was placed in the test box for 10 min, after which the staircases were removed, and the pellets remaining in each staircase were counted, from which the total number of pellets retrieved on each side was calculated.

Before the 6-OHDA lesions, animals were food restricted for 7 days, after which they were submitted to the staircase test for 5 consecutive days. The first two days were training sessions, and the results of the last two days were plotted as pre-lesion baseline values. Afterwards, the animals were returned to a free feeding schedule for one week before surgery, and also during the three weeks of apomorphine testing. Thereafter, the food restriction schedule was reinstated and the test was applied again on several numbers of days after the lesion. The values recorded on the last two dates were plotted as post-lesion baseline values.

2.9. Histology

To assess the extent of 6-OHDA-induced dopamine denervation, the brains of four rats from the HD group were processed for tyrosine hydroxylase (TH) immunohistochemistry. At the end of the experiments rats were deeply anaesthetized and perfused through the ascending aorta with 200 mL of ice-cold phosphate buffer solution (PBS; 0.1 M, pH 7.4) followed by 300 mL of ice-cold paraformaldehyde (4%) in PBS. The brains were removed, postfixed for 2 h in the same fixative at room temperature, and placed overnight in PBS with 15% sucrose at 4 °C. Serial coronal sections (70 μm) of the striatum and substantia nigra were cut using a vibroslicer (Vibratome™) and sequentially placed in multiwells with PBS. The most anterior coronal section was cut at about 500 μm

from the rostral pole of the caudate-putamen (CPu), and six more caudal sections were cut 350 μm apart from each other. The substantia nigra was serially cut in its whole rostro-caudal extent, and alternate sections were immunostained. The selected sections were incubated for 2 h at room temperature with a blocking solution containing bovine serum albumin (1%), triton-X100 (0.3%), and sodium azide (0.0125%) in PBS. Sections were then incubated for 72 h at 4 °C with a rabbit anti-TH polyclonal antibody (Chemicon), at a 1:1000 dilution. The Vectastain ABC kit (Vector) was used thereafter. Sections were washed three times with the blocking solution, and then incubated for 2 h at room temperature with a mouse biotinylated anti-rabbit IgG secondary antibody, diluted 1:500. Next, they were incubated for 2 h with the avidin–peroxidase conjugate and finally developed with a solution of H_2O_2 (0.01%) with 3,3′-diaminobenzidine and cobalt chloride as colour intensifier.

Immunostained sections were mounted onto gelatinised slides and coverslipped, and photographed with a digital camera (Olympus DP11) fixed on a microscope (Olympus SZ11). Digital images were processed with the Adobe Photoshop Elements software to eliminate colour and reduce background. A frame surrounding the CPu or the SN pars compacta was manually placed, and the number of black pixels of the non-lesioned side was counted. Next, the frame was displaced over the lesioned side, approximately in the same corresponding area, and the black pixels were counted there. Both values were used to calculate the percent lesion of the dopamine-denervated side.

2.10. Statistics

In compliance with international policies for the use of animals in neuroscience research, the number of rats used in this study was kept to the minimum necessary to obtain statistically significant results. Results are expressed as means ± SEM. The paired Student's *t*-test was considered appropriate to compare the motor performance between the ipsilateral and the contralateral forepaws in the staircase and adjusting steps tests, since each rat was its own control. For the data obtained in the cylinder test, the changes in motor execution over time were analysed with repeated measures ANOVA, followed by Dunnett's post hoc test to check for statistical significance versus the post-lesion baseline. The significance level was set at 0.05. All statistical analyses were performed using GraphPad Prism version 4.

3. Results

Six rats of the high dose (HD) group and 7 rats of the low dose (LD) group met the criteria of a minimum of 300 contraversive turns/90 min after the third apomorphine challenge, 4 weeks after the 6-OHDA lesions. Animals of the HD group performed 778 ± 108 rotations (range 342–1143), and those of the LD group 525 ± 62 rotations (range 392–825). Fig. 2A illustrates the rotation time-course averages for both groups. The percent loss of TH-immunoreactivity in four rats of the HD group was 94.3 ± 3.9% in the CPu (Fig. 2B), and 92.0 ± 3.5% in the substantia nigra (Fig. 2C).

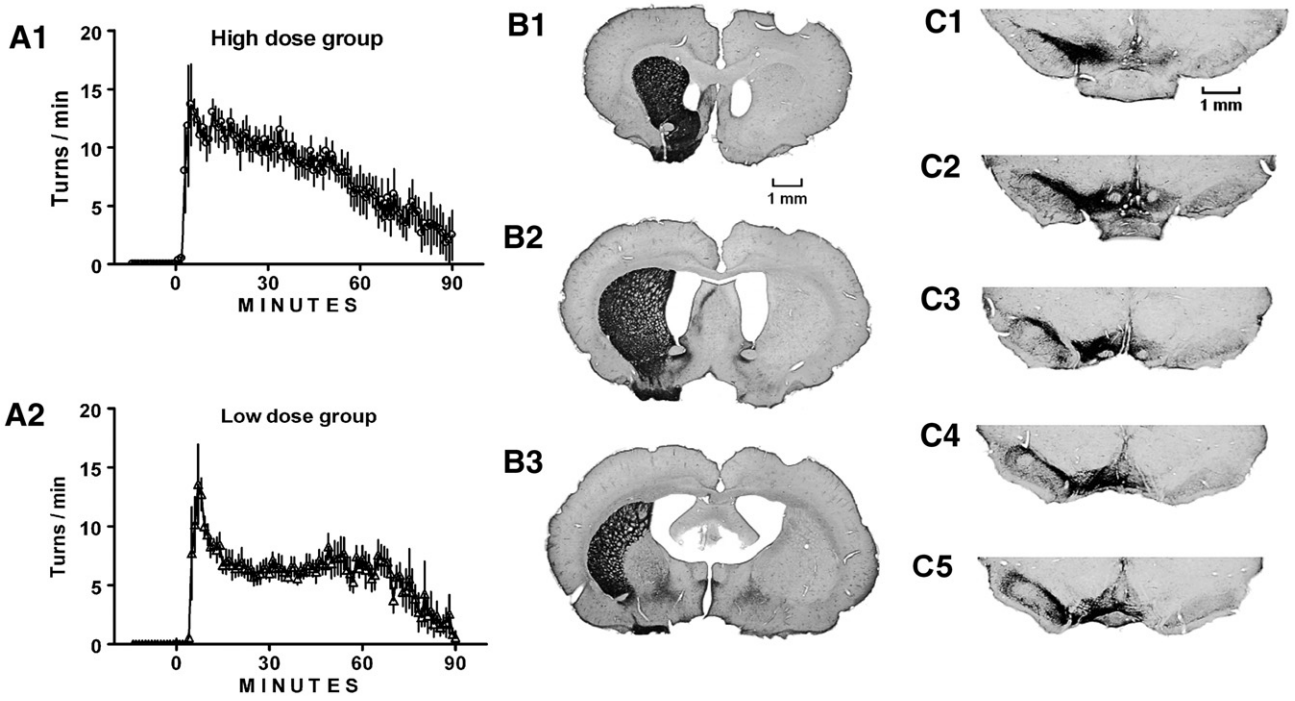

Fig. 2. Assessment of 6-OHDA lesion. (A1–A2) The graphs show the average time course of apomorphine-induced circling 4 weeks after the unilateral lesion with 6-OHDA, in the high dose (A1, $n=6$) and low dose (A2, $n=7$) groups. (B1–B3) Representative coronal sections immunostained for TH at different rostrocaudal levels of the caudate-putamen. (C1–C5) Representative coronal sections immunostained for TH at different rostrocaudal levels of the substantia nigra. All sections are from the same rat. Notice the almost complete lack of TH immunocytochemical staining in the caudoputamen and substantia nigra of the denervated sides.

3.1. Effect of caffeine and THP on the adjusting steps test

During the last day of testing before the 6-OHDA lesions, the animals of the LD group (1 mg/kg caffeine and 0.1 mg/kg THP) performed similar number of steps with the ipsilateral (15.3±0.7, $n=7$) and contralateral (16.0±1.1, $n=7$) forepaws. Seven weeks after the lesion, the contralateral forepaw executed a significantly lower number of steps (4.0±0.8, $n=7$) than the ipsilateral forepaw (15.5±0.7, $n=7$) (Fig. 3A). These values are within the range reported by others (Chang et al., 1999). The impairment of contralateral stepping remained essentially unchanged during the period in which the animals received the aspartame solution wherein drugs were dissolved (Fig. 3A). After 20 days of treatment with the vehicle, the contralateral forepaws still executed fewer steps (4.6±1.2) than the ipsilateral forepaws (14.8±0.5). By contrast, all drug treatments increased the adjusting steps of the contralateral forepaw, gradually approaching the average number of steps of the ipsilateral

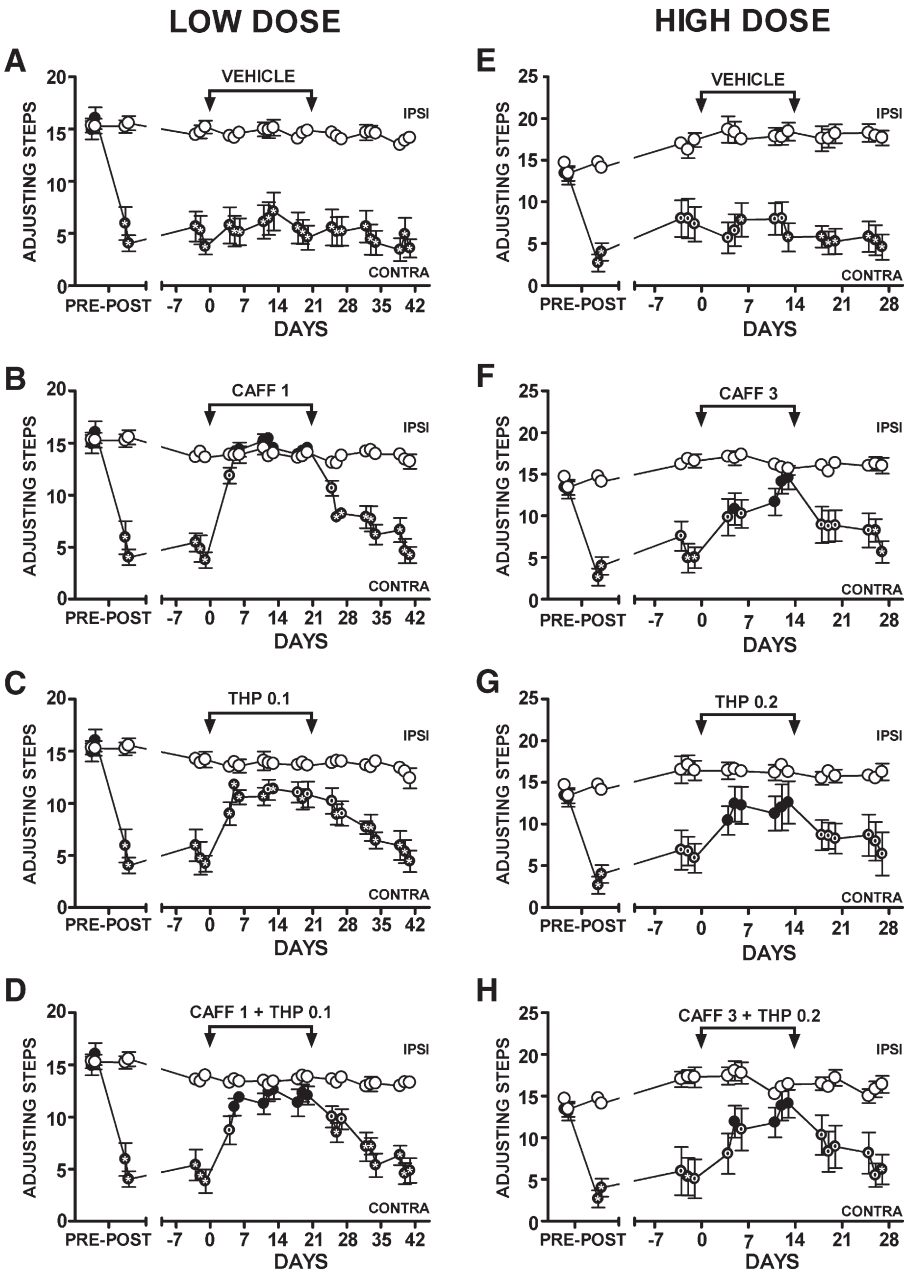

Fig. 3. Time course of the performance in the adjusting steps test during chronic administration of caffeine or THP. Each symbol represents the mean±SEM of the number of steps performed with the ipsilateral (○) and contralateral (●) forepaws by rats in the low dose drug regimen ($n=7$, graphs A to D) and animals in the high dose schedule ($n=6$, graphs E to H). The marked symbols indicate significant differences between contralateral and ipsilateral forelimbs (⊙, $P<0.05$; ✪, $P<0.01$; paired t-test). Compared with pre-lesion values (PRE), the 6-OHDA lesion produced a significant reduction of adjusting steps in the contralateral but not the ipsilateral forelimb (POST). Treatment with the vehicle (aspartame solution, graphs A and E) did not induce recovery in the execution of the contralateral forelimb. In contrast, a daily oral dose of caffeine [CAFF] (graphs B and F), trihexyphenidyl [THP] (graphs C and G), or their combination (graphs D and H) administered for two or three weeks increased the number of adjusting steps performed by the contralateral forelimb. The arrows mark the start and endpoint of drug or vehicle administration. Note that the effect of drugs was gradually reversed once their administration was stopped.

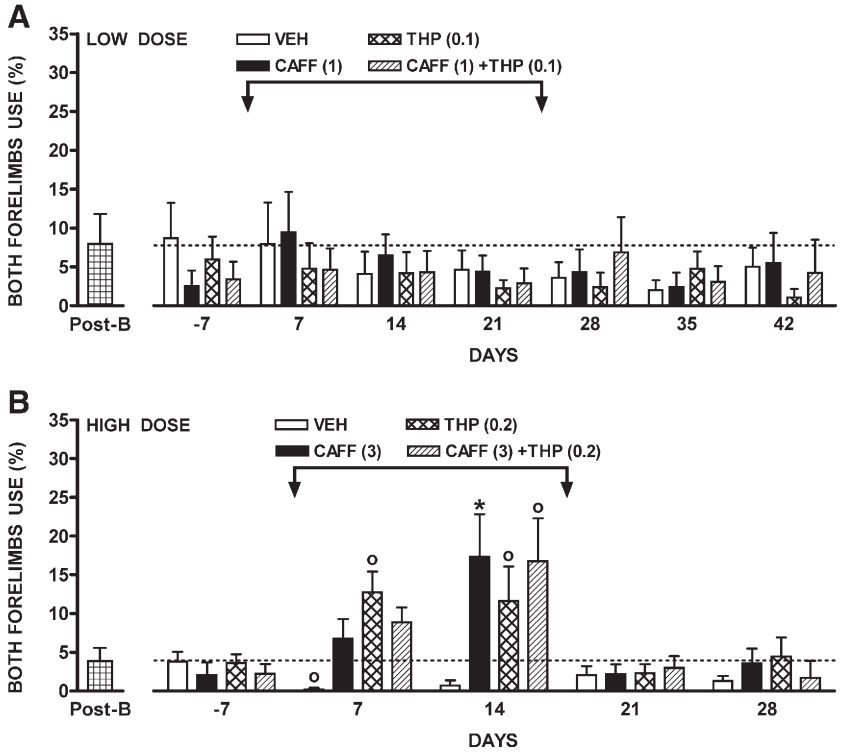

Fig. 4. Chronic treatment with caffeine improves the simultaneous forelimb use in unilaterally 6-OHDA lesioned rats. The number of wall contacts performed simultaneously by the ipsilateral and contralateral forepaws during 5 min inside the cylinder is expressed as a percentage of the total number of paw contacts. Bars represent the mean±SEM of these values. The percent contacts recorded over time during each treatment were analysed with repeated measures ANOVA. In both graphs, the leftmost bar and the broken line represent the average post-lesion baseline (Post-B) against which other means were compared using the post hoc Dunnett's test (○, $P<0.05$, and *, $P<0.01$). Graph A shows the results in the LD group ($n=7$). In these animals, three weeks of daily treatment (p.o.) with the vehicle, caffeine (CAFF, 1 mg/kg), trihexyphenidyl (THP, 0.1/mg/kg) or its combination did not produce a significant effect on the simultaneous forelimb contacts. Graph B summarizes the results in the HD group. During treatment with the vehicle there was a significant effect over time [$F(5,25)=3.053$, $P=0.0273$], with a significant reduction of the wall contacts on the 7th day. Treatment with THP (0.2 mg/kg) caused a significant effect [$F(5,25)=5.618$, $P=0.0013$] manifested by increases on the 7th and 14th days. Caffeine (3 mg/kg) alone produced a significant effect [$F(5,25)=5.347$, $P=0.0018$], but only on the 14th day. Similarly, the combination of caffeine (3 mg/kg) and THP (0.2 mg/kg) also produced a significant increase [$F(5,25)=4.635$, $P=0.0040$] on the 14th day.

forepaw. By the 5th day of treatment with caffeine, at the dose of 1 mg/kg/day, the execution of both forepaws was practically identical: contralateral, 14.1±0.6 steps; ipsilateral, 13.8±0.5 steps (Fig. 3B). Most importantly, this 100% recovery was maintained throughout the remaining 16 days of treatment, and was slowly reversed during the washout period. Treatment with the muscarinic antagonist THP, at the dose of 0.1 mg/kg/day, also produced a sustained recovery of the stepping with the contralateral forepaw (Fig. 3C), but the effect at the 20th day of treatment (10.8±1.2 steps) averaged only 80±9% of the ipsilateral forepaw (13.6±0.5 steps). The combined treatment with caffeine (1 mg/kg/day) and THP (0.1 mg/kg/day) produced the same effect as caffeine alone (Fig. 3D).

During the last day of testing before the 6-OHDA lesions, the animals in the HD group (3 mg/kg caffeine and 0.2 mg/kg THP) performed similar numbers of steps with the ipsilateral (13.4±0.9, $n=6$) and contralateral (13.1±1.0 steps, $n=6$) forepaws. Eight weeks after the lesion the contralateral forepaw executed significantly less steps (4.0±1.1, $n=6$) than the ipsilateral forepaw (14.1±0.5, $n=6$) (Fig. 3A). Again, treatment with the vehicle (aspartame solution) did not reverse the stepping impairment of the contralateral forepaw (Fig. 3E). When caffeine was administered at the dose of 3 mg/kg/day, the number of steps performed with the contralateral forepaw increased, although this effect developed at a slower rate than with the 1 mg/kg dose. However, by the 13th day of treatment the execution of both forepaws was not statistically different: contralateral, 14.6±1.4 steps; ipsilateral, 15.7±0.8 steps (Fig. 3F). At the dose of 0.2 mg/kg/day THP also improved the contralateral stepping (Fig. 3G), but the effect recorded during the 13th day of treatment (12.6±2.6 steps) averaged only 77±16% of the ipsilateral forepaw (16.3±0.7 steps). However, these means were not statistically different. The combined treatment with caffeine (3 mg/kg/day) and THP (0.2 mg/kg/day) did not accelerate the recovery of stepping with the contralateral forepaw, the magnitude and time course of which were similar to those seen with caffeine alone (Fig. 3H).

3.2. Effect of caffeine and THP on the forelimb use asymmetry test

During the 5 min of vertical exploration in the cylinder, the percentage of wall contacts with the forepaw contralateral to the dopamine-denervated side was 0.9±0.6% in the LD group ($n=7$) and 0.4±0.3% in the HD group ($n=6$), which represent significant reductions in comparison with the values recorded

before the 6-OHDA lesions: 23.2±2.9% and 29.1±4.1%, respectively (data not shown in graphs). Similarly, the wall contacts executed simultaneously with both forepaws diminished to 7.9±3.9% in the LD group (Post-B in Fig. 4A) and to 3.9±1.7% in animals of the HD group (Post-B in Fig. 4B), as compared with pre-lesion values of 49.7±5.4% and 59.8±6.0%, respectively. At the same time, the percent use of the ipsilateral forelimb increased from 27.1±3.3% (LD group) and 11.1±3.8% (HD group) before lesion to 91.2±4.3% and 95.7±1.9%, respectively, after dopamine denervation.

The LD regimen produced no significant effects on the independent or the simultaneous forelimb contacts on the cylinder wall (Fig. 4A). In contrast, the higher doses of caffeine (3 mg/kg) and THP (0.2 mg/kg) caused a significant increase of the wall contacts executed simultaneously with both forelimbs (Fig. 4B), which was accompanied by a reciprocal reduction of the wall contacts performed with the ipsilateral forelimb alone (data not shown). Treatment with the vehicle of drugs (aspartame solution) did not reverse the impairment of simultaneous forepaw contacts produced by dopamine denervation (Fig. 4B). Compared with the post-lesion baseline, THP (0.2 mg/kg/day) increased the number of wall contacts by the end of the first and second weeks of treatment. Caffeine (3 mg/kg), either alone or in combination with THP, only induced a significant recovery at the end of the second week (Fig. 4B). Nonetheless, there was no synergism between caffeine and THP to improve the simultaneous forelimb use of rats lesioned with 6-OHDA.

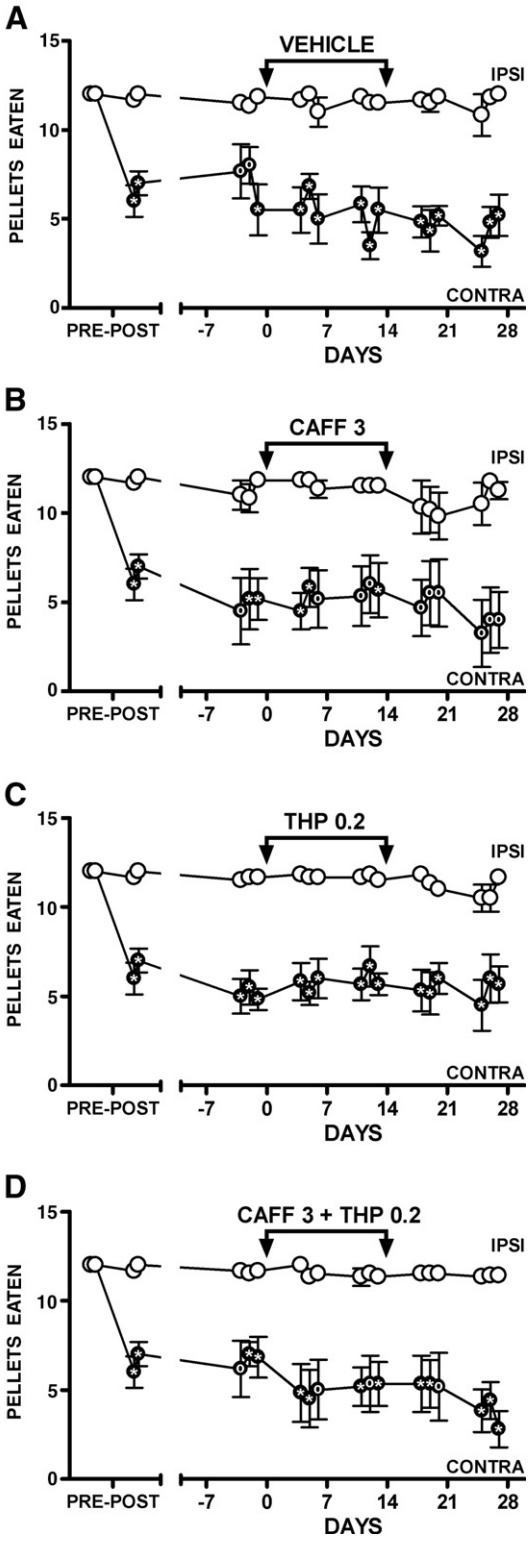

Fig. 5. Chronic treatment with caffeine or THP does not improve the ability of the contralateral forepaw to retrieve food in unilaterally 6-OHDA lesioned rats. The staircase test was applied only to the six rats scheduled for the high dose drug regimen. Each symbol represents the mean±SEM of the number pellets retrieved from the staircase with the ipsilateral (○) and contralateral (●) forepaws during each day of testing. The marked symbols indicate significant differences between the contralateral and the ipsilateral forelimbs (⊙, $P<0.05$; ✪, $P<0.01$; paired t-test). Compared with pre-lesion values (PRE), the 6-OHDA lesion significantly impaired the ability of the contralateral forepaw to retrieve food (POST). The number of pellets recovered by the contralateral forelimb did not increase during treatment with the vehicle (A), 3 mg/kg/day caffeine (B), 0.2 mg/kg/day trihexyphenidyl (C), or their combination (D). The arrows mark the start and endpoint of drug or vehicle administration.

3.3. Effect of caffeine and THP on the paw-reaching staircase test

After the 6-OHDA lesions, the number of food pellets retrieved in 10 min by the contralateral forepaw was reduced to 7.0±0.7 ($n=6$), which represents 58±6% of pre-lesion values (12±0 pellets eaten, Fig. 5A). This percent decrease is similar to the values reported by other authors (Olsson et al., 1995). As shown in Fig. 5B–D, during the two weeks of treatment with caffeine (3 mg/kg/day) or THP (0.2 mg/kg/day), either alone or in combination, there was no reversal of the grasping impairment produced by the unilateral dopamine denervation.

4. Discussion

The main finding of the present study was that chronic oral treatment with low doses of the non-selective adenosine antagonist caffeine (1–3 mg/kg/day) reversed the motor deficits of hemiparkinsonian rats in the adjusting steps test. Besides, chronic treatment with 3 mg/kg caffeine significantly improved the use of the affected forepaw in the cylinder test. In both cases, the recovery of motor function did not develop tolerance during the whole treatment period, and the observed effects were fully

reversible when caffeine was discontinued. Similarly, low chronic doses of the muscarinic antagonist trihexyphenidyl (0.1–0.2 mg/kg/day) enhanced the performance of the forepaw on the hemiparkinsonian side in the cylinder and the adjusting steps tests, although the effect in this last test was less intense than that produced by caffeine. Nevertheless, neither caffeine nor THP improved the grasping in the staircase test, and their combined administration showed no synergism to improve the motor execution in any of the tests.

Although the unilateral 6-OHDA lesion of the nigrostriatal dopaminergic pathway in rats is accepted as a useful method for the rapid assessment of potential antiparkinsonian drugs (Kaakola and Teravainen, 1990; Cenci et al., 2002), there is evidence that the predictive validity of this model depends on which behaviour is selected as a measure of therapeutic outcome (Lindner et al., 1996; Lundblad et al., 2002). It has been emphasized that the most clinically relevant models would be those that measure motor impairments that have the greatest similarity with the neurological manifestations of Parkinson's disease, and with enough sensitivity to give a quantitative assessment of the motor recovery induced by drugs (Cenci et al., 2002; Lindner et al., 1996; Lundblad et al., 2002). The results of the present study clearly show that chronic treatment of hemiparkinsonian rats with low doses of caffeine (1 and 3 mg/kg) and THP (0.1 and 0.2 mg/kg) produced a sustained improvement of the motor function in the stepping test, which has been compared to the "standing pull test" used by neurologists in diagnosing Parkinson's disease (Schallert and Tillerson, 2000). Notably, the recovery of forepaw stepping induced by both doses of caffeine was quite similar in magnitude to the effect produced by 6.25 mg/kg L-DOPA plus 50 mg/kg benserazide evaluated with this same test (Chang et al., 1999). These findings suggest that the stepping test measures a motor deficit that is highly sensitive to the motor activating effects of antiparkinsonian compounds. In the cylinder test, however, only the higher doses of caffeine (3 mg/kg) and THP (0.2 mg/kg) caused a partial recovery of the forelimb use, whereas both drugs were ineffective to reverse the impairments of reaching and grasping movements evaluated in the staircase test. Similar to caffeine and THP, neither apomorphine nor selective D_1 or D_2 dopamine agonists are able to reverse the impairment of forepaw reaching in hemiparkinsonian rats (Olsson et al., 1995). These observations indicate that the staircase test does not have enough sensitivity to evaluate the full therapeutic potential of antiparkinsonian drugs. Taken together, the results of the present study suggest that low doses of caffeine should be useful to reduce the akinesia or to increase the postural stability in parkinsonian subjects. Although this proposal awaits confirmation in primate models or in clinical trials, the present findings add further support to the idea that evaluation with a battery of clinically relevant tests is the only way to fully assess the therapeutic potential of antiparkinsonian drugs (Lindner et al., 1996; Lundblad et al., 2002).

In this study, only rats performing at least 300 full contraversive rotations in 90 min after the third apomorphine (0.25 mg/kg) challenge were used for the experiments. This number of turns is compatible with striatal dopamine depletions greater than 90% (Schwarting and Huston, 1996). However, the number of rotations performed by rats of the HD group was on average higher than that of rats in the LD group. Although comparison of the means did not reach significance ($P=0.059$, unpaired t-test), it was close, suggesting that some rats of the HD group had greater dopamine depletion than rats in the LD group. Since the motor activating effect of caffeine is largely dependent on a tonic activation of striatal D_2 dopamine receptors (Zahniser et al., 2000), it is possible that subtle differences in the residual dopamine content would influence its effects in hemiparkinsonian rats. This could explain the faster recovery (1 week) of contralateral stepping in rats treated with 1 mg/kg caffeine, compared with animals receiving the 3 mg/kg dose (2 weeks).

It is worth mentioning that the contraversive rotation induced by high doses of caffeine (10–30 mg/kg) in hemiparkinsonian rats is totally dependent on the previous stimulation of supersensitive striatal dopamine receptors by a dopamine agonist (Fenu and Morelli, 1998). Since selection of successfully lesioned animals in our study involved testing with three doses of apomorphine, we can not rule out the possibility that this non-selective dopamine agonist could have enabled the motor improvement induced by low doses of caffeine (1–3 mg/kg) in the stepping and cylinder tests. If this hypothesis proves to be true, priming with dopamine agonists would be necessary to observe the therapeutic effects of low doses of caffeine in parkinsonian patients.

Pharmacokinetic studies in rats have shown that following an acute 5 mg/kg dose, caffeine reaches a steady plasma concentration of about 4 μg/mL (Lau and Falk, 1994), roughly equivalent to 20 μmol/L. Therefore, it is reasonable to assume that chronic treatment with the lower doses of caffeine (1–3 mg/kg/day) used in the present study produced plasma concentrations below 20 μmol/L. Under these conditions, the molecular targets in the rat brain to which caffeine binds with greater affinity are the A_1 ($K_D=20$ μmol/L) and A_{2A} ($K_D=8.1$ μmol/L) adenosine receptor subtypes (Daly et al., 1999; Fredholm et al., 1999). Most evidence points to the notion that the motor stimulant activity induced by caffeine in rodents and other species is mainly mediated by blockade of the A_{2A} receptors of the brain (Ferré et al., 2001; Fisone et al., 2004; Fredholm et al., 1999). Since only the A_{2A}, but not the A_1 antagonists, are still able to produce motor activation following striatal dopamine depletion (Ferré et al., 2001), it can be assumed that the improvement of motor performance of the forepaw contralateral to the 6-OHDA lesioned side of rats chronically treated with low doses of caffeine was mediated through blockade of adenosine A_{2A} receptors in the basal ganglia nuclei. This idea is supported by studies showing that the CPu is one of the brain structures that is most sensitive to the effects of low doses of caffeine, since a single parenteral dose of 1 mg/kg, which blocks 32% of striatal adenosine A_{2A} receptors (El Yacoubi et al., 2001), increases the striatal glucose uptake by about 25% above basal levels (Nehlig, 1999; Nehlig and Boyet, 2000). These studies correlate with our finding that chronic oral treatment of hemiparkinsonian rats with this same dose of caffeine produced

a significant reversal of akinesia of the forelimb contralateral to the dopamine-denervated side in the adjusting steps test. At the dose of 1 mg/kg caffeine alone produced a full recovery in the number of steps of the contralateral forelimb when the rat was displaced laterally. In the cylinder test, however, only the 3 mg/kg/day dose caused a significant increase in the number of wall contacts performed simultaneously with both forepaws. It is possible that the latter effect was mediated by recruitment of other basal ganglia structures, since a 2.5 mg/kg i.v. dose of caffeine enhances glucose utilization not only in the CPu, but also in the globus pallidus (Nehlig, 1999). This last structure, which is a key nucleus in the control of motor behaviour, receives afferents from the striatal GABAergic neurons expressing a large number of adenosine A_{2A} receptors (Ferré et al., 2001; Fredholm et al., 1999), whose blockade with selective A_{2A} antagonists reverses the increase of GABA release produced by striatal dopamine denervation (Ochi et al., 2000). In the staircase test, though, neither of the caffeine doses used improved the retrieval of food with the contralateral forepaw. This suggests that the blockade of adenosine A_{2A} receptors by caffeine is not enough to compensate for the loss of fine motor control during grasping behaviour that develops after dopamine denervation of the CPu.

In the circling behaviour model, doses of caffeine of 40 mg/kg produce strong and sustained tolerance (Casas et al., 1999), but doses close to the range of daily human consumption (2.5 and 10 mg/kg) only produce a trend to induce tolerance, that does not reach statistical significance (Yu et al., 2006). The results of the present study clearly show that chronic oral treatment with doses of caffeine between 1 and 3 mg/kg/day fully restores the motor performance of hemiparkinsonian rats in the adjusting steps test without the development of tolerance for up to 3 weeks. This effect was gradually reversible upon suspension of the drug, indicating that it was dependent on the presence of caffeine. This finding suggests that the beneficial effects of caffeine on parkinsonian patients could be sustained during chronic dosage.

The muscarinic antagonist THP (0.1–0.2 mg/kg/day) produced a significant improvement in the stepping of the forepaw contralateral to the 6-OHDA lesion, although its magnitude was only about 77–80% of the effect produced by caffeine. In the cylinder test only the highest dose of THP increased the use of the contralateral forelimb. It is likely that the ability of THP to improve the motor execution of the contralateral forelimb in hemiparkinsonian rats was mediated through blockade of the striatal muscarinic receptors, since clinical and experimental evidence indicates that the loss of dopaminergic innervation leads to an increase of cholinergic transmission in the CPu (De Boer et al., 1993; Fahn et al.,, 1990; Stoof et al., 1992).

Although previous studies showed that the combination of low doses of caffeine and THP acts in synergy to restore the motor function in pharmacological models of parkinsonism (Moo-Puc et al., 2003, 2004), this drug combination did not produce further improvement of the motor performance in hemiparkinsonian rats. This absence of synergism could be due to neuroadaptive mechanisms that occur only after the permanent loss of striatal dopamine innervation, such as the enhanced expression of mRNA for enkephalin in the striato-pallidal neurons, and the downregulation of mRNA for substance P in the striatonigral neurons (Gerfen et al., 1990).

In conclusion, this study shows for the first time that low chronic doses of caffeine, within the range of habitual human consumption, produce a sustained improvement in the use of the contralateral forelimb in unilaterally 6-OHDA dopamine denervated rats, without the development of tolerance. It also shows that low chronic doses of THP produce a similar though less efficient effect. Even when the combination of caffeine and THP showed no synergism in this preclinical model, the results suggest that low doses of caffeine deserve further testing in primate models or in controlled clinical trials to fully assess their efficacy in the treatment of Parkinson's disease.

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