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The dopamine agonist piribedil with L-Dopa improves attentional dysfunction : relevance for Parkinson's disease

Nathalie Turle-Lorenzo, Béatrice Maurin, Carole Puma, Claire Chezaubernard , Philippe Morain, Christelle Baunez , André Nieoullon and Marianne Amalric

Laboratoire de Neurobiologie de la Cognition UMR 6155 CNRS -Université de Provence (N. T.L, B. M., C.P.,C.B.,M.A.)

Institut de Biologie du Développement de Marseille-Luminy (IBDML),UMR 6216 CNRS -Université de la Méditerranée, Marseille, France (A.N.)

Institut de Recherches Internationales Servier, Courbevoie, France (C.C., P. M.)

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Corresponding author : Marianne AMALRIC

Laboratoire de Neurobiologie de la Cognition

UMR 6155 CNRS -Université de Provence, Case C

3 Place Victor Hugo

13331 Marseille cedex 3

France

Phone: (33) (0)4 88 57 68 73

Fax: (33) (0)4 88 57 68 72

Email: marianne.amalric@up.univ-mrs.fr

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Abbreviations :

Piribedil = [(methylenedioxy-3,4 benzyl)-4 pyperazinyl-1]-2 pyrimidine)

L-DOPA = L-3,4-dihydroxyphenylalanine

6-OHDA = 6-hydroxydopamine

MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

RT = reaction time

Abstract

Cognitive deficits are often associated with motor symptoms in Parkinson's disease. This study investigates the ability of piribedil [(methylenedioxy-3,4 benzyl)-4 pyperazinyl-1]-2 pyrimidine), a D2/D3 dopamine receptor agonist with antagonist activity at α_{2A}-adrenoceptors, to restore motor and attentional deficits in nigrostriatal 6-hydroxydopamine-lesioned rats. Subjects were trained to depress a lever, detect a stimulus occurring after variable foreperiods and release the lever quickly afterwards. Striatal DA depletions produce deficits in the timing of foreperiods and prolong reaction times. While a subchronic treatment with piribedil (0.1 to 2 mg/kg) is not effective, a dose of 0.3 mg/kg administered for 3 weeks significantly reverses the akinetic deficits produced by the striatal dopamine depletion and progressively improves attentional deficits. When co-administered with the dopamine prodrug L-3,4-dihydroxyphenylalanine (L-DOPA 3 mg/kg), piribedil (0.3 mg/kg) promotes a rapid and full recovery of preoperative performance. These results suggest that administration of L-DOPA in combination with piribedil in a chronic treatment as either initial or supplemental therapy for Parkinson's disease might improve cognitive functions while reducing the risk for motor complications.

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Introduction

Following the discovery that patients with Parkinson's disease (PD) suffer from a dopamine deficiency in the basal ganglia, the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA) has been successfully administered to supplement the depleted DA stores. The development of motor fluctuations and abnormal involuntary movements (dyskinesia), is however a common side-effect of the long-term treatment with L-DOPA. DA receptor direct agonists are now commonly used as initial therapy to limit the exposure to L-DOPA and delay the onset of dyskinesia (Jenner, 1995).

Among the current DA agonists acting directly on synaptic receptors in the striatum, piribedil ([1-(3,4-methylenedioxybenzyl)-4-(2-pyrimidinyl)piperazine] is a centrally acting drug, that shows selectivity for both D2 and D3 dopaminergic receptors and significant antagonistic action on α2A adrenergic receptors (Millan et al., 2001). In the rat brain piribedil revealed comparable affinities for the dopamine D3 and the D2-like receptors and very low affinity for the D1-like receptors (Millan et al., 2002). The anti-akinetic activity of piribedil was first reported in reserpinized rats and in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned primate (Smith et al., 1996; Smith et al., 2002). Its efficacy in controlling motor symptoms of Parkinsonian patients with an incidence of dyskinesia lower than that occurring in patients treated with L-DOPA was recently shown in PD patients (Montastruc et al., 1999 ; Tan et al., 2003). The chronic treatment with piribedil for a 6-month period in combination with L-DOPA is well tolerated and significantly improves motor symptoms in non fluctuating Parkinsonian patients (Ziegler et al., 2003). In addition to the classical motor symptoms, several cognitive deficits are observed in non-demented patients with PD, even at the early stages of the disease (Dubois and Pillon, 1997). The pattern of cognitive impairment includes deficits of executive functions, such as planning and working memory (Brown and Marsden, 1990; Owen, 2004) and attentional deficits resembling that produced by

frontal-lobe damage (Lewis et al., 2003). In the MPTP-treated marmosets, it has been reported that, in parallel to its efficacy in reducing behavioural motor deficits, piribedil induced increased vigilance and awareness as compared to L-DOPA (Smith et al., 2002). Piribedil, probably through its α2A adrenergic antagonist properties, was shown to increase acetylcholine release both in frontal cortex and in the hippocampus (Smith et al., 2002; Gobert et al., 2003). This action may be related to its facilitatory influence upon cognitive function.

Besides motor improvement, the possibility to restore cognitive functions (memory, reaction time, speed of information processing, etc.) with DA agonists in PD represents therefore a new challenge. The aim of the present study was thus to test the effects of piribedil on motor and cognitive deficits in a rat model of early PD. The nigrostriatal dopamine pathway was damaged bilaterally by an intrastriatal infusion of the neurotoxin 6-hydroxydopamine (6-OHDA), that induces a progressive and selective loss of DA neurons. It has long been shown that DA depletion in the striatum produces profound deficits in reaction time (RT) paradigm in rats (Spirduso et al., 1985; Amalric and Koob, 1987; Brown and Robbins, 1991; Amalric et al., 1995; Smith et al., 2000; Courtiere et al., 2005). We have previously found that rats, trained to quickly react to a visual cue occurring after a variable interval, are slower to react to the cue and sometimes impaired in the timing of the various intervals depending on the extent of striatal DA denervation (Amalric et al., 1995). These deficits may be related to some extent to the akinesia, assessed by increased RTs (Berry et al., 1999), and attentional dysfunction that are commonly seen in Parkinsonian patients (Gauntlett-Gilbert and Brown, 1998). In the present study, we have tested the efficacy of piribedil and L-DOPA alone or in conjunction to restore motor and cognitive deficits in 6-OHDA-lesioned rats trained in the RT task.

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Methods

Experiment 1 : locomotor activity

Animals

Male Wistar rats weighing 280-300 g on arrival from Iffa-Credo (Lyon, France) were housed in groups of two per cage with food and water available ad libitum and maintained in temperature-controlled conditions with an alternating 12h light – 12h darkness cycle (lights on at 07.00 h.). Behavioral measurements were conducted during the animals' light cycle. All procedures were in strict accordance with the French "Ministère de l'Agriculture et de la Pêche", Décret n° 87-848, October 19, 1987 and to the European Communities council directive of November 24th, 1986 (86/609/EEC).

Apparatus

A bank of 16 individual wire (top, floor, and front door) and Plexiglas (side walls) photocell cages was used to measure the locomotor activity. Each cage (40 × 25 × 23 cm) was fitted with two parallel horizontal infrared beams, 1 cm above the floor, located across the long axis of the cage (Imetronic, Pessac, France). Beam interruptions were accumulated over 1 min intervals and recorded in bins of 1 min by an one-line input to a microcomputer. The animals were familiarized with the experimental cages during a 3h session, one day before the test session. On the day of testing, the spontaneous locomotor activity was monitored for 90 min prior to drug treatment. Four groups of animals (n=3 per group) received different doses of piribedil (0, 1, 3 or 10 mg/kg) in a different order of injection following a pseudo-random Latin-square design. Injections were performed every 3 days and the animals were placed immediately afterwards in the locomotor activity cages for a total duration of 180 min. After a wash-out period of 10 days, selected animals received either NaCl 0.9 % (n=4) or piribedil 1.0 mg/kg (n=8) once a day for 5 consecutive days and the locomotor activity was recorded for 180 min.

Statistical analysis

Data analysis was carried out using a two-factor analysis of variance (ANOVA). When animals were injected according to a Latin square design, we first analysed the effects of the order of injections between the different groups. If no significant effect, the differences between the various doses over time were subjected to a two-factor ANOVA, with the different groups (doses of piribedil) as the independent factor and time as the repeated measure. When the ANOVA revealed a significant effect, *post hoc* comparisons were carried out using the Newman-Keuls test. The significance level was taken to be $P < 0.05$.

Experiment 2: reaction time task

Animals

Male Wistar rats (Charles River, France) weighing 115-120 g at the start of the experiment, were housed in groups of two and maintained on a 12-hour light/dark cycle with lights on at 7 a.m. In the reaction-time task, rats were initially food deprived for 24 hours at the start of training and subsequently food restricted to 15-20 g of laboratory chow per rat per day for the duration of the experiment to maintain them at 80-85% of the free food body weight. Water was available *ad libitum*.

Apparatus and operant procedure

Experiments were conducted in standard experimental chambers (23 x 22 x 30 cm; Campden Instruments, Cambridge, UK) placed in sound attenuated cubicles. The boxes were controlled and the data collected on line by a PC computer and laboratory interface (Paul Fray, Inc., Cambridge, UK). A retractable lever, located 5 cm above the grid floor and 4 cm below the cue light (2.8 W bulb), was

extended immediately after the animals were placed in the experimental chambers. After completion of a successful trial, the food pellet was delivered to the food magazine in less than 1 sec. The temporal resolution of the instrumental setup was 10 ms. Animals were initially trained to lever press for a 45-mg food pellet (P.J. Noyes Company, Inc., Lancaster, NH) on a schedule that provided one food pellet for every lever press (fixed ratio-1 reinforcement schedule). A force of 0.8 N on the lever was required to operate the switch closure. After the rats successfully responded for more than 100 pellets, they were trained to hold down the lever until the onset of a cue-light located above the lever. To receive a food pellet during this phase of training, rats had to wait for the cue-light and then release the lever with no time limit. The interval between lever press and the cue-light onset was then progressively increased by steps of 50 ms for up to 1250 ms, after 5 consecutive correct trials. At the final step, the intervals were fixed and randomized between four foreperiods (500, 750, 1000 and 1250 ms) to maintain a high level of attention to the cue onset and prepare the motor response. Reaction time (RT) was measured in ms between the presentation of the cue-light and the release of the lever. The RT restriction was then progressively decreased from 2000 to 600 ms over 40 to 50 additional sessions. Each session ended after 100 trials, which animals typically achieved within 10-15 min during the pre-lesion testing period. *Correct responses* were those in which the rats released the lever with RTs below the 600-ms time limit. *Incorrect responses* were not rewarded and divided into two categories. If the lever was released prior to the presentation of the light-cue, it was recorded as an incorrect response and termed a *premature response*. Similarly, if the lever was released with RTs above 600-ms restriction time, it was recorded as an incorrect response and was termed a *delayed response*. Mean RTs averaged 300 ms at the final part of the test.

Bilateral striatal dopamine lesions

Animals were anesthetized by an intramuscular injection of xylazine (156 mg/kg) and ketamine (100 mg/kg) and placed in a stereotaxic instrument (David Kopf Instruments) with the incisor bar positioned – 3.0 mm under the interaural line for surgical procedures based on coordinates of the stereotaxic atlas Paxinos and Watson (Paxinos et al., 1985). Lesioned animals received a bilateral injection of 6-OHDA hydrochloride (Sigma Aldrich, Lyon, France) (4 µg/µl, 3 µl per side) in the striatum at the following coordinates : anteroposterior (AP) +0.2 mm ; lateral(L) ±3.5 mm, dorsoventral (DV) – 4.8 mm (from skull) according to the bregma. The sham control group received the vehicle alone (ascorbate solution , 0.1 mg/ml) in the dorsal striatum. The infusion was made with a micropump over 9 min using a 10 µl Hamilton microsyringe, connected by a Tygon tubing fitting to the 30 gauge stainless steel injector needles. After surgery rats were allowed a 7-day recovery period.

Drugs

Piribedil (Trivastal, Servier, Paris, France) was dissolved in 0.9 % saline and injected intraperitoneally (i.p.) in a volume of 1 mL/kg. L-DOPA methylester (Sigma Aldrich, Lyon, France) was dissolved with a DOPA decarboxylase (benserazide,15 mg/kg) (Sigma Aldrich, Lyon, France) in a 0.9 % saline solution and injected i.p. in a volume of 1 mL/kg.

Experimental procedure

After training lasting on average 2 to 3 months, rats underwent surgical procedure (6-OHDA lesions). Behavioral testing resumed on post-operative day 8 and the 6-day per week testing schedule continued for 4 additional weeks.

Dose response of piribedil : After the recovery period, the effects of 6-OHDA lesions were tested for 6 sessions (e.g. from day 9 to 14 after surgery) and the subjects (n=20) were then divided into 4 subgroups. The different groups were injected with one of 4 doses of piribedil (0, 0.1, 0.3 or 1.0 mg/kg ip, n=5 per dose) and tested 1 hour later in the RT task. This procedure allows the drug to reach its maximal effect in the brain. Piribedil was tested in a sub-chronic treatment of 6 injections on post-operative days 15 to 20. After a 3 days wash-out period, the same groups were injected with higher doses of piribedil (2, 6 and 20 mg/kg) for 6 additional sessions from post-operative days 25 to 30. A sham-operated group of rats (n=5) received vehicle injections (NaCl 0.9 %) and was tested in the same conditions from days 9 to 30 post-surgery.

Chronic treatment with piribedil or L-DOPA : After a 7-day recovery period, the 6-OHDA lesion or surgery effects were tested over 6 sessions. The animals (sham n=32, 6-OHDA n=60) were then divided into 4 subgroups receiving one of the different treatments every day for 3 weeks (from days 16 to 40 postsurgery) : *sham groups* injected with NaCl 0.9% (n=12); piribedil 0.3 mg/kg (n=6), L-DOPA 3.0 mg/kg (n=7), co-injection with piribedil 0.3 + L-DOPA 3.0 mg/kg (n=7) and *lesion groups* injected with NaCl 0.9 % (n=22), piribedil 0.3 mg/kg (n=18), L-DOPA 3.0 mg/kg (n=7), piribedil 0.3 mg/kg + L-DOPA 3.0 mg/kg (n=13). The dose of 0.3 mg/kg piribedil was selected on the basis of the results obtained in the first experiment. The dose of 3 mg/kg L-DOPA was selected on the basis of previous studies showing that this dose induces no dyskinetic effects over a 3 weeks treatment (Henry, 1999) and reduces 6-OHDA lesion-induced deficits in the same task (Turle-Lorenzo et al., 2005). The subjects received the co-injection of L-DOPA 3 mg/kg (with benserazide 15 mg/kg) and piribedil 0.3 mg/kg and 1 hour later were tested in the RT task.

Histology

At the end of the experiment, animals were killed by decapitation. The brains were then removed and frozen to –80°C. Coronal 10 µm tissue sections were cut at –20°C using a microtome cryostat (Leica CM3050) at the level of the striatum.

The binding of [^{3}H]-mazindol to dopamine uptake sites in the striatum was measured according to the procedure described by Javitch (Javitch et al., 1985). Briefly, sections were air dried and rinsed for 5 min at 4°C in 50 mM Tris buffer with 120 mM NaCl and 5 mM KCl. They were then incubated for 40 min with 15 nM [^{3}H]-mazindol (NEN DuPont ; specific activity 17 Ci/mM) in 50 mM Tris buffer containing 300 mM NaCl and 5 mM KCl added with 0.3 mM desipramine to block the noradrenalin transporter. Nonspecific binding was determined by incubating some sections in the same solution plus 30 mM benztropine. Sections were rinsed twice for 3 min in the incubation medium without mazindol and for 10 sec in distilled water and were air dried. Autoradiographs were generated by apposing the sections to ^{3}H-sensitive screen (Raytest, Courbevoie, France) for 7 days and were further quantified with a beta imager (Fuji-Bas 5000).

The estimation of the surface of bilateral striatal 6-OHDA lesions was determined by delimitating the extent of the lesioned areas in each hemisphere. The neuronal loss was measured in the lesioned areas by quantifying grey levels which were converted to optical density (OD) using external standards (calibrated density step tablet; Kodak). Since no difference was found in either the surface or the OD measured on each side of the brain, the values obtained were averaged for statistical analysis. The mean OD of the lesioned areas was then compared with control OD measured on the same striatal region in sham-operated animals and expressed as a percentage of control values.

Data and Statistical Analysis

The number of correct, premature, delayed responses were collapsed across each session by block of 6 days (e.g. weeks). The data were analyzed over time (before and after 6-OHDA lesions and during drug treatments : vehicle, piribedil, L-DOPA or co-administration of piribedil and L-DOPA) using an overall two-way analysis of variance (ANOVA), with one between-subject factor (GROUP) and one within-subject factor (WEEK). As previously found, depending on the extent of striatal DA denervation produced by 6-OHDA infusions, the increase of RTs and delayed responses after the cue may be associated or not with an increase of premature responses before the cue onset, reflecting impulsivity and attentional dysfunction in timing the various interval preceding the cue (Amalric et al., 1995). Chronic piribedil or L-DOPA treatment was thus separately analysed in the group exhibiting delayed responding to the cue with no premature responding (named "*akinetic group*") and in the group expressing both deficits (named "*attentional group*"). One-way ANOVA followed by post hoc tests (Fisher's PLSD test) were used for multiple pairwise comparisons within each group when appropriate. All statistical analyses procedure were performed using Statview 5.0 program (Abacus concept). RTs above 100 ms (corresponding to real detection of the cue in contrast to coincident lever release) and below 800 ms were analyzed in selected sessions of the pre- and post-operative period (before and after drug treatment) in order to further examine the individual distribution of RTs. The distribution of RTs was plotted as a percentage of the total number by 50 ms bins (frequency) ranging from 100 to 800 ms and averaged for all subjects of a group. To analyze the delay-dependent speeding of RT, RTs were averaged as a function of the various foreperiods in all subjects during a pre- and postoperative session before and after pharmacological treatment and were submitted to a one-way ANOVA with two within-subject factors (pre/post-lesion and the 4 foreperiods). In addition, the distribution of premature responses (expressed as a percentage of the total number of premature responses by 50 or 250 ms-bin) was further analyzed with

regards to the various foreperiods in representative pre- and postoperative sessions (before and after chronic treatment). Data were analyzed using a two-way ANOVA with two within-subject factors: pre/postlesion sessions and foreperiods.

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Results

Experiment 1 : Locomotor response to piribedil

As shown on Fig. 1, following administration piribedil transiently decreased rat's locomotor activity within the first 30 min with no further changes over the 180 min testing period. Following a non significant effect of the order of injections of piribedil at different doses (P=0.4), the ANOVA testing the effects of piribedil over time demonstrated a significant main effect of time ($F_{17,51}$ = 31.02, P<0.01) and a significant dose × time interaction ($F_{51,748}$= 4.43, P<0.01). Subsequent analysis performed during the exploratory phase of the test (e.g. first 30 min following piribedil injection, see inset) showed a clear depressant short-term effect whatever the dose tested. Therefore in the following experiments, piribedil effects on RT performance were tested one hour after systemic injection. Data showed that during the period of the testing, there was no tolerance to the repeated injection of piribedil since a sub-chronic treatment with a dose 1.0 mg/kg for 5 consecutive days did not modify the locomotor response which was depressed in the same proportion on the 5th day in comparison to the first one (data not shown).

Experiment 2 : reaction time performance

Sub-chronic treatment with piribedil. At completion of the training phase, all animals reached a pre-operative level of 65-70 correct responses with incorrect responses distributed among 25-30 premature and 5 delayed trials per 100-trial session. There was no significant difference in the pre-lesion baseline values for any parameter of motor performance among the groups. As illustrated in Figure 2, striatal 6-OHDA lesions impaired RT performance as indicated by a significant decrease in the number of correct responses when compared with preoperative levels (main lesion effect $F_{1,16}$ = 22.51, p<0.01 PLSD

Fisher's test). Comparisons between groups showed no significant difference on correct, premature or delayed responses, before and after 6-OHDA lesions. Therefore they were pooled together. The decrease of correct performance was dependent upon variations of premature and delayed responses as revealed by a significant overall effect of lesion on these parameters (main lesion effect $F_{1,16}$ = 5.6 and 18.67 for premature and delayed responses respectively; all $p<0.05$). Piribedil treatment significantly modified the number of correct, premature and delayed responses over time ($F_{3,48}$= 24.09, 5.24 and 9.6 respectively; all $p<0.01$) with no significant differences among subgroups treated with different doses. At low doses, 0.1 to 1 mg/kg piribedil had no significant effect on the deficits produced by the 6-OHDA lesions since all the animals exhibited the same level of premature and delayed responses than observed during the first postoperative week (Fig. 2). At the higher doses of 6 and 20 mg/kg, piribedil markedly impaired RT performance by enhancing the 6-OHDA-induced increase in the number of delayed responses ($p<0.05$ Fisher PLSD test). Piribedil 20 mg/kg decreased the number of correct responses to 24 trials/session as early as the second day of injection. This effect lasted throughout the 6 days treatment (not shown). In addition, a tendency to increase premature responses was also observed at that dose. Since piribedil at doses above 2 mg/kg produced deleterious effects on RT performance and lower doses were not active when tested in a sub-chronic 6-day treatment, the duration of treatment was then increased for up to 3-week of daily injection with a low dose of piribedil (0.3 mg/kg).

Chronic treatment with piribedil or L-DOPA. As found in a previous experiment (Amalric et al., 1995), 6-OHDA lesions disrupted the performance by increasing both delayed and premature responding in 63 % of the subjects (n=38/60), while a selective effect on delayed responses was observed in the remaining 37 %. The effects of piribedil or L-DOPA were then separately analysed in these two groups (e.g. "attentional" versus "akinetic", see methods).

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Effects of piribedil or L-DOPA as a single treatment. The striatal DA depletion severely impaired RT performance by dramatically increasing the number of premature responses (Fig. 3A). No recovery of preoperative levels was observed for the entire 4-week testing period (baseline versus post, 1st, 2nd and 3rd weeks for correct responses, $p<0.05$ Fisher PLSD test after significant ANOVA). In addition, the number of delayed responses progressively increased over time and remained significantly higher than before lesion ($p<0.05$ baseline versus weeks 1 and 3). As shown in Fig. 3B, piribedil 0.3 mg/kg gradually reduced the number of premature responses over time with no recovery of preoperative level of performance, however (pre versus post only, $p<0.05$, Fisher PLSD test after significant ANOVA). In contrast, piribedil increased the number of delayed responses at the second week of treatment as compared to baseline ($p<0.05$, Fisher PLSD test). A similar pattern of responses was observed after L-DOPA 3.0 mg/kg chronic treatment, although the decrease of premature responses at the last week of treatment (albeit non significant) led to an improvement of the correct performance over time (3rd week versus post only, $p<0.05$, Fisher PLSD test after significant ANOVA). L-DOPA had no effect on the number of delayed responses at any time post-lesion.

Effects of piribedil and L-DOPA co-administration. As illustrated in Fig. 4, there was a significant improvement of RT performance in rats treated with a combination of the two drugs at same doses. The number of correct responses gradually returned to preoperative levels over time (postlesion versus 3rd week, $p<0.01$; prelesion versus 1st and 2nd weeks only, $p<0.05$). The increased number of premature responses was significantly reduced at the 1st week of co-treatment and remained significantly lower than post level for the total duration of the testing period (post-hoc test following significant one-way ANOVA, postlesion versus 1st, 2nd and 3rd weeks, $p<0.05$). Piribedil and L-DOPA co-administration

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had a biphasic effect on the number of delayed responses, enhancing the deficit produced by 6-OHDA lesions at short-term while reversing it at the last week of treatment (2^{nd} week significantly different from prelesion and 3^{rd} week, $p<0.01$, Fig. 4). Piribedil or L-DOPA injected alone or in combination had no effect on performance of sham-operated animals (Table 1).

Effects of piribedil and L-DOPA co-administration on attentional deficits. To further investigate the nature of the premature responses produced by the 6-OHDA lesion in relation to the variable foreperiods, we analyzed the distribution of these responses (plotted by 50 ms or 250 ms bins) in a pre- and post-operative session, at day 13 post-lesion, in comparison to the last day of piribedil and L-DOPA co-treatment (Fig. 5A and B). According to classical models of motor preparation using simple RT procedure, when variable foreperiods are equiprobable and randomized within a series of trials, the conditional probability of the cue onset increases as time elapses leading to an increased level of motor preparation, resulting in faster RTs as a function of foreperiod duration (Brown and Robbins, 1991). Furthermore, the distribution of the premature responses was not linear after the second foreperiod (750 ms) but rather presented peaks of responses after the value of the cue onset (Fig. 5A). This suggests that the animals were able to use the knowledge of the variable foreperiods to prepare their response. In addition, logically the proportion of premature responses increased as a function of time (significant main effect of foreperiods ($F_{3,27}=14.71$, $p<0.05$, Fig. 5B). 6-OHDA lesions disrupted this pattern and the proportion of premature responses was found to be similar in the different foreperiods (significant interaction surgery x foreperiods $F_{3,27}=3.39$, $p<0.05$). Interestingly, the lesions did not affect the knowledge of the duration of the foreperiod values since the peaks were preserved. As illustrated in Fig. 5B, the pattern of premature responses increasing as a function of foreperiods length in a preoperative session ($F_{3,36}=8.64$, $p<0.05$, significant difference in foreperiods 3 and 4 in comparison to foreperiods 1

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and 2, $p<0.05$ Fisher PLSD test) was disrupted by the lesion ($F_{3,36}=1.2$, ns, Fig. 4B). After chronic treatment with piribedil and L-DOPA, the pattern of preoperative premature responses was fully recovered (significant difference in foreperiods 3 and 4 in comparison to foreperiods 1 and 2, $p<0.05$ Fisher PLSD test after significant treatment x foreperiod interaction: $F_{3,36}=12.1$, $p<0.01$).

Effects of piribedil and L-DOPA co-administration on motor preparation. The effects of 6-OHDA lesions were further investigated on the motor preparatory mechanisms by examining the variation of RTs with regards to the different foreperiods (Fig. 6). Preoperatively, RTs were found to be faster as the preparatory level increases (overall main effect of foreperiod $F_{3,27}=22.21$, $p<0.01$). RTs were found to be significantly shorter after foreperiods 3 and 4 as compared with the first foreperiod, ($p<0.05$ PLSD Fisher test, after significant ANOVA $F_{3,36}=4.41$, $p<0.01$) suggesting that the animals have used the conditional probability of cue occurrence to prepare their response. 6-OHDA lesions disrupted this pattern (non significant treatment x foreperiods interaction $F_{6,54}=0.9$, Fig. 6). In contrast, a significant delay-dependent speeding of RTs was found after co-treatment with piribedil and L-DOPA in the last session of testing (day 36 postlesion $F_{3,36}=6.8$, $p<0.01$). The mean RTs in the two longest foreperiods were found to be significantly faster than in the shorter foreperiods ($p<0.05$, PLSD Fisher test) .

Effects of piribedil on akinesia. As illustrated in figure 7A, 6-OHDA lesioned animals injected with vehicle exhibited a significant decrease in the number of correct responses in the first postoperative week as compared to preoperative levels (mean correct 64.3+/-2.9) and remained at a level of 57 +/- 3.3 correct responses throughout the 4-week testing period ($F_{8,32}= 5.7$, $p<0.01$). The decreased number of correct responses was mainly due to a significant increase in the number of delayed responses ($F_{8,32}=$ 6.69, $p<0.01$) with no change in premature responding (NS, not shown). The effects of the lesions were

long lasting since no recovery was observed for up to 40 d post-lesion (pre vs all postoperative weeks, $p<0.05$ for delayed responses Fisher's PLSD test, Fig.7A). Piribedil at a low dose of 0.3 mg/kg normalized the number of delayed responses over time (post-hoc following significant one-way ANOVA: postlesion vs 3rd week, $p<0.05$, presurgery vs 1st week only, $p<0.01$, Fisher's PLSD test). As illustrated in Fig. 7B, the RTs distribution showed a shift to the right of the curve towards longer values after lesion and piribedil 0.3 mg/kg induced a shift back towards preoperative values. The increase of mean RTs induced by 6-OHDA lesions (pre vs post, $p<0.05$ Fisher's PLSD test after significant ANOVA) was reduced by a 3-week treatment with piribedil (Fig. 7C).

Histological results. Examination of ^{3}H-mazindol binding to DA uptake sites was undertaken to assess the extent and the localization of DA terminals loss in the striatum in comparison to sham-operated controls (Fig. 8). Intrastriatal 6-OHDA infusions produced in general a lesion restricted to the dorsal part of the striatum sparing the fibers located along the lateral ventricle. There was no loss of ^{3}H-mazindol binding in the nucleus accumbens and the olfactory tubercle. Animals found to be inconsistently depleted (e.g., asymmetric lesion or low level of depletion n=9) were excluded from the statistical analysis. These animals exhibited either no deficits or a transient increase of delayed responses which rapidly recovered within the first week of testing. As classically reported, treatment with 6-OHDA or other neurotoxins (MPTP, rotenone, MDMA) that damage DA neurons produces variability in the level of DA depletion (Schwarting and Huston, 1997). In the present study, we therefore performed quantitative analyses in selected animals, belonging to the *attentional* (n=10) or *akinetic* (n=11) group, in order to verify whether the size or intensity of the lesion could explain the behavioral differences. The optical density (OD) of ^{3}H-mazindol labeling was quantitatively analyzed and compared at three different anteriority levels (A 1.2 mm, A 0.36 mm and A –0.72 mm) within and outside the core of the lesion. The decrease of ^{3}H-

mazindol labeling in the core of the lesion averaged 87 % in the *attentional* group and 58 % in the *akinetic* group in comparison with sham-operated controls (Fig. 8 and 9A). A non-significant 15-22 % decrease in the surrounding striatum was also found in both groups (Fig. 9B). A significant interaction between groups and anteriority levels was found ($F_{2,38}$= 8.79, $p<0.01$) showing that the extension of the DA depletion was different rostrocaudally. The main difference was observed in the most rostral levels where a 41 % decrease of labeling was found in the *akinetic* group as compared to a 85 % decrease in the *attentional* group ($p<0.01$, simple ANOVA). At the bregma level of anteriority, on sections close to the injection site, the decrease of labeling reached 67 % and 89 % for the *akinetic and attentional* groups, respectively, with no significant difference between the 2 groups. The DA depletion extended caudally to a similar extent in the 2 groups (65 and 86 % decrease, respectively) (Fig. 9A). These results were similar to the measure of the endogenous striatal DA contents assessed by HPLC in a previous study showing a 74 % depletion of tissue levels of DA in the posterior striatum and 53 % depletion in the anterior striatum (Amalric et al., 1995). In addition, a significant difference was found between the two groups on the surface of the core of the lesion at these three different levels of anteriority. As illustrated in Fig. 9B, the surface of the lesion core in the *attentional* group was 1.4 mm^2 rostrally and increased up to 2.2 and 1.8 mm^2 caudally. In contrast, the lesion in the *akinetic* group (Fig. 8C) was virtually absent rostrally (0.09 mm^2) and reached a surface of 0.9 and 0.8 mm^2 at caudal levels ($p<0.05$ at the three levels, non paired t test after significant ANOVA $F_{1,19}$= 18.8, $p<0.01$).

Discussion

In the present study, we showed that the D2/D3 dopamine receptor agonist / α2A antagonist piribedil is able to counteract the akinetic deficits produced by partial striatal bilateral 6-OHDA lesions in rats trained in a RT task similar to that used to assess motor initiation deficits in parkinsonian patients (Gauntlett-Gilbert and Brown, 1998 ; Muller et al., 2000). Low dosage of 0.3 mg/kg of piribedil was effective in recovering preoperative performance, provided the animals were chronically injected with the drug. In conditions of extensive 6-OHDA lesions impinging on the rostral regions of the striatum, additional deficits were observed on motor readiness and time estimation that were reduced by piribedil 0.3 mg/kg and fully reversed with a co-administration of L-DOPA at a dose of 3.0 mg/kg.

The positive effects of piribedil in reversing 6-OHDA lesions induced akinesia and bradykinesia are in line with recent studies conducted in patients with PD showing a significant improvement of the motor symptoms (Montastruc et al., 1999; Ziegler et al., 2003; Simon et al., 2005). Here we found that piribedil is efficient at a low dose (0.3 mg/kg) under a chronic treatment. It is unlikely that these anti-akinetic effects are due to a nonspecific increase of motility since doses previously found to increase locomotor activity and stereotyped behaviors in rats ranged from 50 to 100 mg/kg (Dourish, 1983). In contrast, lower doses cause depression of activity when tested immediately after injection (Simon et al., 2005). This is consistent with the idea that DA agonists at low doses preferentially activate DA autoreceptors situated on the cell bodies and fibers of neurons in the nigrostriatal system. The inhibition of DA synthesis and turnover may account for the increased number of delayed responses following piribedil treatment in the attentional group and for the decreased exploratory behavior in the photocell cages.

DA agonist drugs used to control PD vary in their profile of action on D1/D2 and D3 receptors. Piribedil binds preferentially to D2 and D3 receptors and has no significant affinity for the D1 receptors (Millan et al., 2001). Since the blockade of D2 receptors with selective antagonists (raclopride, eticlopride) but not of the D1 or D3 receptor subtypes in rats produced similar RT impairment as those observed here after 6-OHDA nigrostriatal lesions in the same task (Smith et al., 2000), it is tempting to suggest that the reversal of the akinetic deficits is due to piribedil action on striatal D2 receptors. In marmoset monkeys, piribedil improves parkinsonism induced by the neurotoxin MPTP (Smith et al., 1996; Smith et al., 2002) and the improvement of motor function is primarily attributed to activation of postsynaptic receptors in the basal ganglia (Jenner, 1995).

More interestingly is the findings that piribedil treatment may also affect non-motor deficits in the same task. In addition to the execution of rapid movements, successful completion of the RT task requires that the rats are attentive to the presentation of the light cue. The loss of control over the 'hold period' of the lever when attending to the light cue after 6-OHDA lesions, illustrated by the increased number of premature responses, therefore suggest a loss of attentional control and a disruption of motor preparation. This leads to impulsive responsiveness and loss of inhibitory control which is a general feature observed in human and non-human primates with frontal lesions (Fuster, 1997; Evenden, 1999). Moreover, there is a considerable overlap between the cognitive deficits observed following damage to the frontal lobes and those described in some patients with PD (Brown and Marsden, 1990; Owen, 2004; Lewis et al., 2003; Berry et al., 1999). Our results indicate that animals with DA denervation spreading to the most rostral regions of the striatum innervated by the prefrontal cortex (e.g. the *attentional group*), have lost their ability to time the different foreperiods and are therefore unable to use the increasing probability of the cue occurrence to prepare their response to the cue. As a consequence the delay-

dependent speeding of RTs or "motor readiness" appears to be disrupted by the largest DA depletion in the striatum as previously found by others (Brown and Robbins, 1991). Consistent with the idea that a dysfunction of the complex loop between the anterior part of the striatum (e.g. caudate nucleus in human) and the prefrontal cortex underlies the cognitive deficits of PD, excitotoxic lesions to the prelimbic-infralimbic cortical areas in rats have been found to produce similar impairment of motor preparatory processes in the same RT task (Risterucci et al., 2003). The progressive reversal of these deficits by piribedil may be due to its action on D3 and/or α2-adrenergic receptors localized in the frontal cortex. Recent in vitro and in vivo investigations of piribedil properties have indeed reported a significant affinity for α2-adrenergic receptors in addition to the DA D2/D3 receptors (Millan et al., 2001). As an α2-adrenergic receptor antagonist, piribedil was found to enhance frontocortical release of acetylcholine (Gobert et al., 2003 ; Newman-Tancredi et al., 2002). Basal forebrain cholinergic neurons are well known to be involved in attentional processing and the loss of these neurons from the Parkinsonian brain may contribute to attentional dysfunction. In PD patients, cholinergic treatments have had only a limited success in treating executive and attentional deficits, while some of these are ameliorated with naphtoxazine, a compound with α1-adrenoceptor agonist-like activity (Bedard et al., 1998).

Selective antagonists of α2-adrenergic receptors, such as atipamezole or idazoxan, attenuate motor symptoms (circling behavior) in unilateral 6-OHDA lesioned rats (Chopin et al., 1999), while in 1-methyl-4-phenyl-1'2'3'6-tetrahydropyridine (MPTP)-treated monkeys they facilitate the action of L-DOPA (Bezard et al., 1999) and attenuate dyskinesia (Henry et al., 1999 ; Grondin et al., 2000). None of these studies, however, have investigated the action of these drugs on cognitive deficits in animal models of PD. We found here that repeated administration of piribedil in adjunction to L-DOPA in rats bearing bilateral 6-OHDA lesions attenuate the attentional dysfunction and reverse the akinetic deficits. Whether

the improvement of cognitive deficits involves a selective action on α2-adrenergic receptors, ultimately modulating cholinergic activity, or on DA D3 receptors still remains to be clarified.

Clinical studies recently showed the efficacy of piribedil in the treatment of PD in monotherapy or in conjunction with L-DOPA (Ziegler et al., 2003). When tested in MPTP-treated marmosets, long-term treatment (30 days) with piribedil or L-DOPA produces equivalent reversal of motor deficits over the course of the study. In contrast to L-DOPA, however, piribedil produces a significantly lower degree of dyskinesia (Smith, 1996; Smith, 2000; Smith, 2002).This may relate to the recent findings that blocking α2-adrenergic receptors in PD patients improves L-DOPA-induced dyskinesia without the reappearance of parkinsonian symptoms (Colosimo and Craus, 2003). In the present study, the improvement of 6-OHDA –induced deficits was not associated with abnormal dyskinetic movements at any time post-lesion. The initial alteration of the delayed responses in the first weeks of co-treatment, however, is likely to be due to a selective effect of dopamine agonists injected at low dose. This was attributed to a presynaptic action on dopaminergic terminals therefore primarily reducing DA synthesis. In line with this, L-DOPA treatment was found to produce sedation in *de novo* parkinsonian patients (Andreu et al., 1999; Muller et al., 2000). In the long-term, the chronic treatment with piribedil may produce tolerance to these immediate effects through desensitisation of DA receptors and ultimately regulate DA function on postsynaptic receptors.

The effects of piribedil on cognitive functions have not yet been tested in parkinsonian patients but in elderly patients. Nagaraja et al. (Nagaraja and Jayashree, 2001) demonstrated, after a randomized, double-blind clinical trial that piribedil improves global cognitive function in patients with mild cognitive impairment. In young healthy volunteers, piribedil improves alertness and speed

information processing (Schuck et al., 2002). Altogether, these results indicate that piribedil in combination with L-DOPA may have beneficial effects on cognitive function in the early stages of PD.

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Footnotes:

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Legends for Figures

Figure 1 : Effect of the dopamine D2/D3 receptor agonist piribedil on spontaneous locomotor activity. Three groups of animals received different doses of piribedil (0, 1, 3 or 10 mg/kg) in a different order of injection. All the animals were placed in the photocell activity cages immediately after the injection and locomotor activity was recorded for a total duration of 180 min.

* Significantly different from controls, $p < 0.01$, Fisher PLSD test after significant ANOVA.

Figure 2 : Effects of piribedil administered to 6-OHDA lesioned rats in the reaction time task. Effects are measured on correct, premature and delayed responses before (open bar) and after 6-OHDA lesion (solid bar). The effects are measured during various blocks of six sessions corresponding to : one block before surgery (pre), one block from days 9 to 14 after lesion (post), and two blocks during piribedil subchronic treatment. Low doses (0.1, 0.3 and 1 mg/kg, n=5 each) are tested from days 15 to 20 (first block) and higher doses (2, 6 and 20 mg/kg) from days 25 to 30 (second block) after lesion. The vertical axis gives the mean number of responses ± SEM per block for the three variables measured.

* significant difference from preoperative performance ($p<0.05$; Fisher PLSD test after significant ANOVA). $^{\#}$significant difference from postoperative performance ($p<0.05$; Fisher PLSD test).

Figure 3 : Effects of chronic treatment with vehicle (n=7) (A), piribedil (0.3 mg/kg, n=9) (B) or L-DOPA (3 mg/kg, n=7) (C) in lesioned animals exhibiting attentional and akinetic deficits in the RT task. The effects are illustrated on the mean number ±SEM of correct, premature and delayed responses averaged by blocks of 6-session for the pre- and postoperative periods and during the 3 weeks treatment.

* significant difference from preoperative performance ($p<0.05$; Fisher PLSD test after significant ANOVA). # significant difference from postoperative performance ($p<0.05$; Fisher PLSD test).

Figure 4 : Effects of a co-administration of piribedil (0.3 mg/kg) and L-DOPA (3 mg/kg) in lesioned animals (n=10) exhibiting attentional and akinetic deficits in the RT task. The effects are illustrated on the mean number ±SEM of correct, premature and delayed responses averaged by blocks of 6-session for the pre- and postoperative periods and during the 3 weeks treatment.

* significant difference from preoperative performance ($p<0.05$; Fisher PLSD test after significant ANOVA). # significant difference from postoperative performance ($p<0.05$; Fisher PLSD test).

¤ significant difference from the 1st week of treatment ($p<0.05$; Fisher PLSD test).

Figure 5: Effects on the pattern of premature responses produced by 6-OHDA lesions and by the co-administration of piribedil (0.3 mg/kg) and L-DOPA (3 mg/kg). A. Distribution of the premature responses (lever release before the cue-light onset) during a pre (day – 2 : open area), a postoperative (day 13 : solid area) and a post-treatment session (day 36 : shaded area). The mean percentage of premature responses is plotted as a function of time in 50 ms-bin.

B. Mean percentage of premature responses is averaged for each 250 ms-period preceding the cue onset.

* Significant difference from foreperiods 1 and 2 ($p<0.01$; Fisher PLSD test after significant ANOVA).

Figure 6 : Effects of 6-OHDA lesion and piribedil + L-DOPA chronic treatment on reaction times. Mean RTs are plotted as a function of the various foreperiods preceding the cue light onset. Mean RTs were measured during three representative sessions: a preoperative day (day -2) and two postoperative days

(days 13 and 36) corresponding to the lesion effect without treatment in comparison with the end of chronic treatment, respectively.

* significant difference from foreperiod 1 ($p < 0.01$; Fisher PLSD test after significant ANOVA).

Figure 7 : Effects of chronic treatment with piribedil in animals exhibiting akinetic deficits in the reaction time task. A. The effects are illustrated on the mean number ±SEM of correct and delayed responses for the pre- and postoperative periods and during the 3 weeks of chronic treatment in 6-OHDA lesioned animals treated with vehicle (n= 9) or piribedil (0.3 mg/kg, n= 9).

B. Upper graph : RTs distribution is illustrated before (open bars, pre : day – 3) and after striatal 6-OHDA lesion (solid bars, post : day 10). Lower graph : RTs distribution after 3 weeks of daily treatment with piribedil (shaded bars, day 42) in comparison to preoperative distribution (open bars). Each bar represents the RT frequency (percentage) by 50ms-bin from 100 to 800 ms.

C. Mean RTs ± SEM in ms are illustrated before 6-OHDA lesion (pre : day-3), after lesion (post : day 10) and on day 40 postlesion corresponding to the third week of treatment.

* significant difference from preoperative performance ($p<0.05$; Fisher PLSD test after significant ANOVA). # significant difference from postoperative performance ($p<0.05$; Fisher PLSD test).

Figure 8 : Binding of [^{3}H]-mazindol to dopamine uptake sites at striatal level. Photomicrographs comparing the level of [^{3}H]-mazindol labeling in striatal sections from a control subject (A) and representative subjects of the *attentional* (B) and the *akinetic* group (C). The lack of mazindol binding (dashed points) in B and C shows the different extent of 6-OHDA lesions in the dorsal striatum measured at different anteriority levels (from A 1.7 to – 0.6 mm related to bregma). Scale bar =1 mm.

Figure 9 : Quantitative analysis of the effects produced by 6-OHDA infused in the striatum within (A) and around (B) the core of the lesion measured at three different anteriority levels related to bregma (A 1.2-1.08 mm, A 0.36-0 mm and A – 0.72-0.96 mm). The effects are compared in the two lesioned groups : *akinetic* (shaded bar, n=11) and *attentional* (solid bar, n=10) related to the sham control group (open bar, n=9). * significant difference from control group, ($p<0.01$, ¤ significant difference between *akinetic* and *attentional* group ($p<0.01$).

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Table 1. Effects of chronic treatment with vehicle, piribedil (0.3 mg/kg), L-DOPA (3.0 mg/kg) alone or in combination on correct, delayed and premature responses in sham-operated animals.

treatment	trials	Pre	Post	Week 3
vehicle (n=12)	correct	69 ± 3.89	70 ± 3.14	71 ± 3.67
	premature	26 ± 4.34	24 ± 3.22	23 ± 3.76
	delayed	5 ± 1.52	6 ± 1.44	6 ± 1.43
piribedil (n=6)	correct	72 ± 3.89	71 ± 3.85	74 ± 3.99
	premature	22 ± 3.58	23 ± 4.31	20 ± 4.72
	delayed	6 ± 1.40	6 ± 2.84	6 ± 2.43
L-Dopa (n=7)	correct	68 ± 5.56	67 ± 5.68	64 ± 5.45
	premature	25 ± 5.34	27 ± 7.74	31 ± 5.24
	delayed	7 ± 2.58	6 ± 3.01	5 ± 1.61
Piribedil + L-Dopa (n=7)	correct	70 ± 5.57	67 ± 5.92	75 ± 6.09
	premature	23 ± 4.33	27 ± 3.76	18 ± 5.04
	delayed	5 ± 1.96	4 ± 0.85	6 ± 1.61

The values correspond to the mean number of correct, delayed and premature responses during different sessions: one preoperative (pre, day - 2); one postoperative (post) at day 13 after the lesion and on the last postoperative day (+ 36) recorded immediately after vehicle, piribedil, L-DOPA or piribedil + L-DOPA injections.

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Figure 1

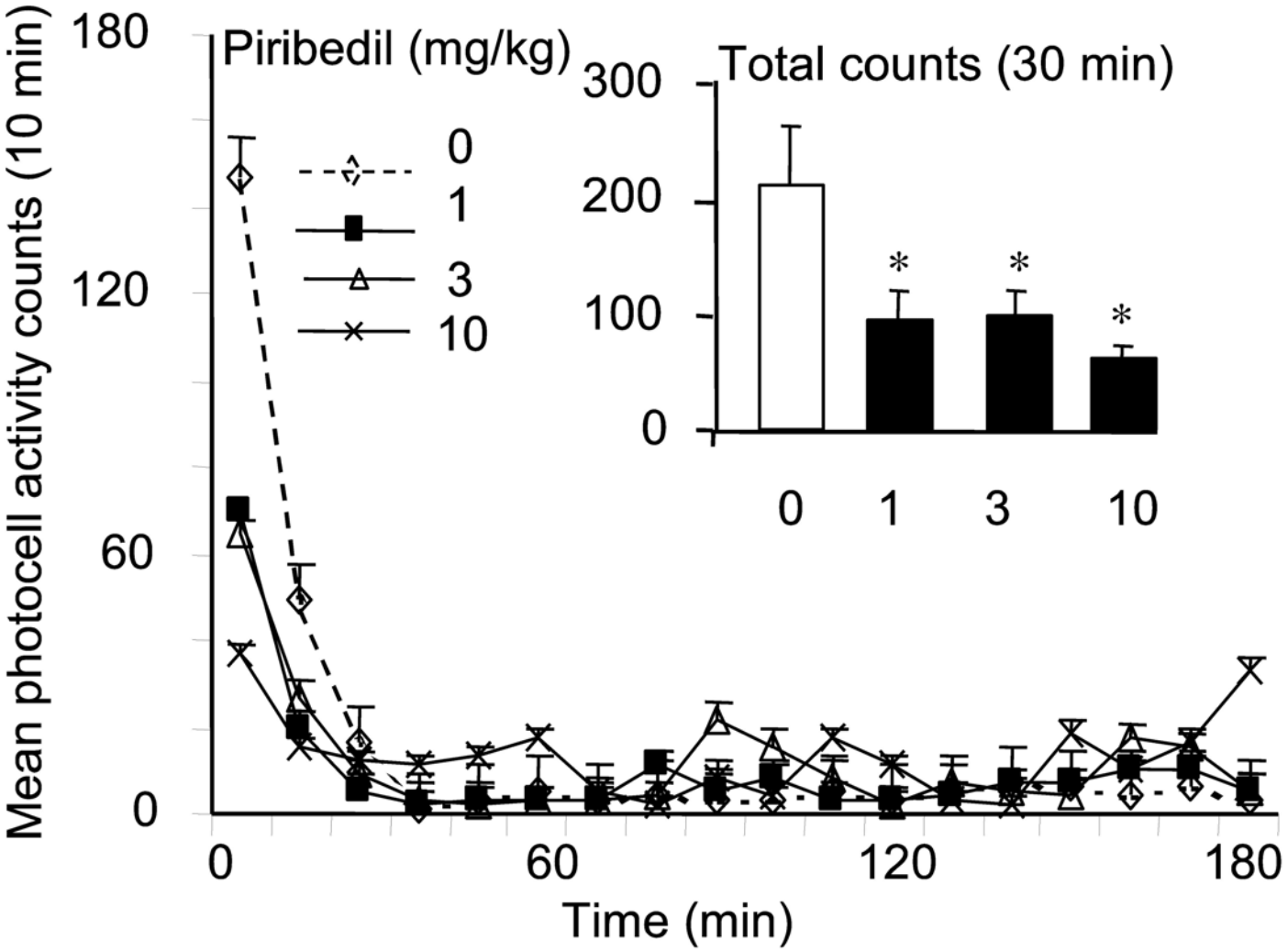

Figure 2

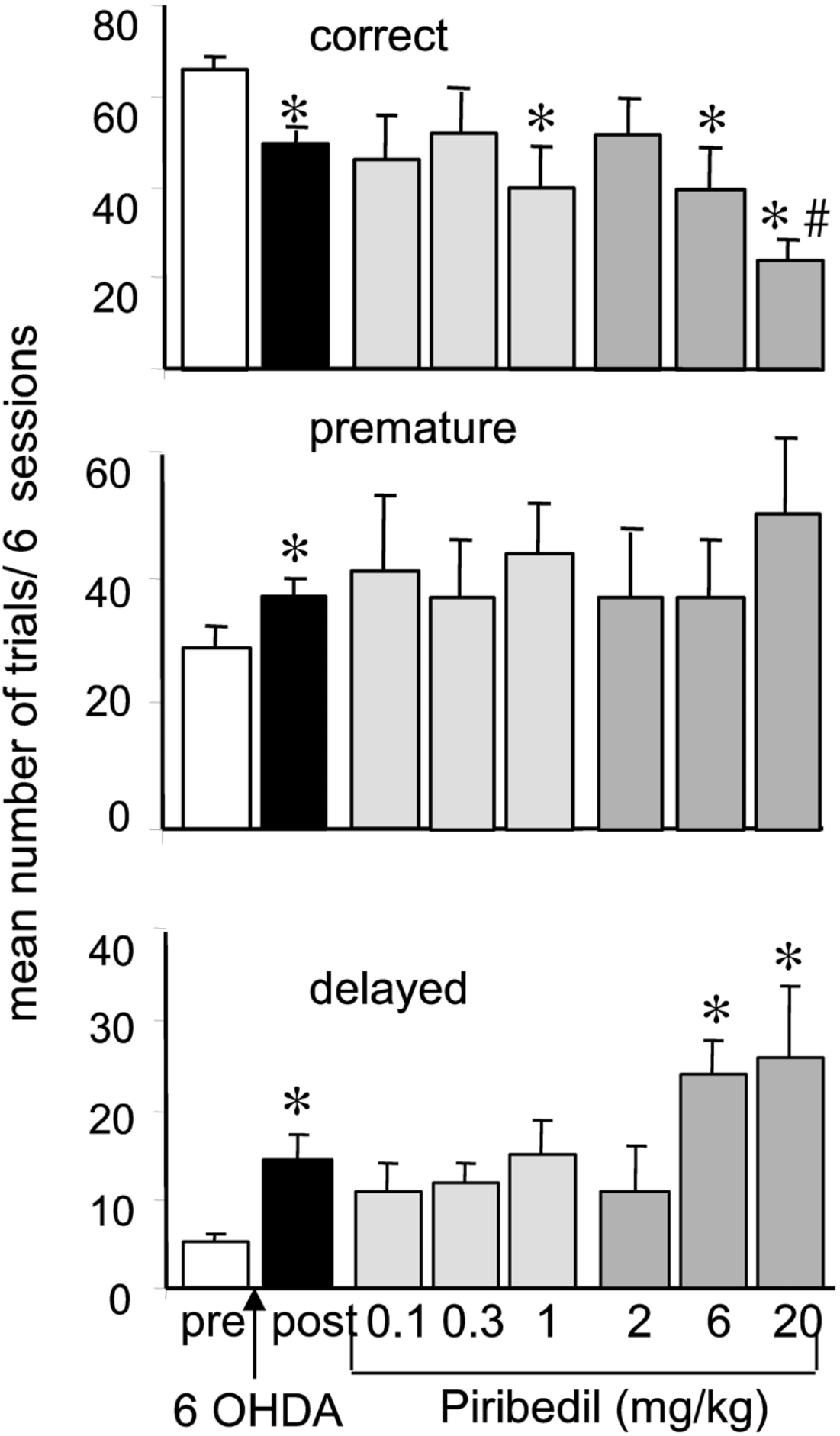

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Figure 3

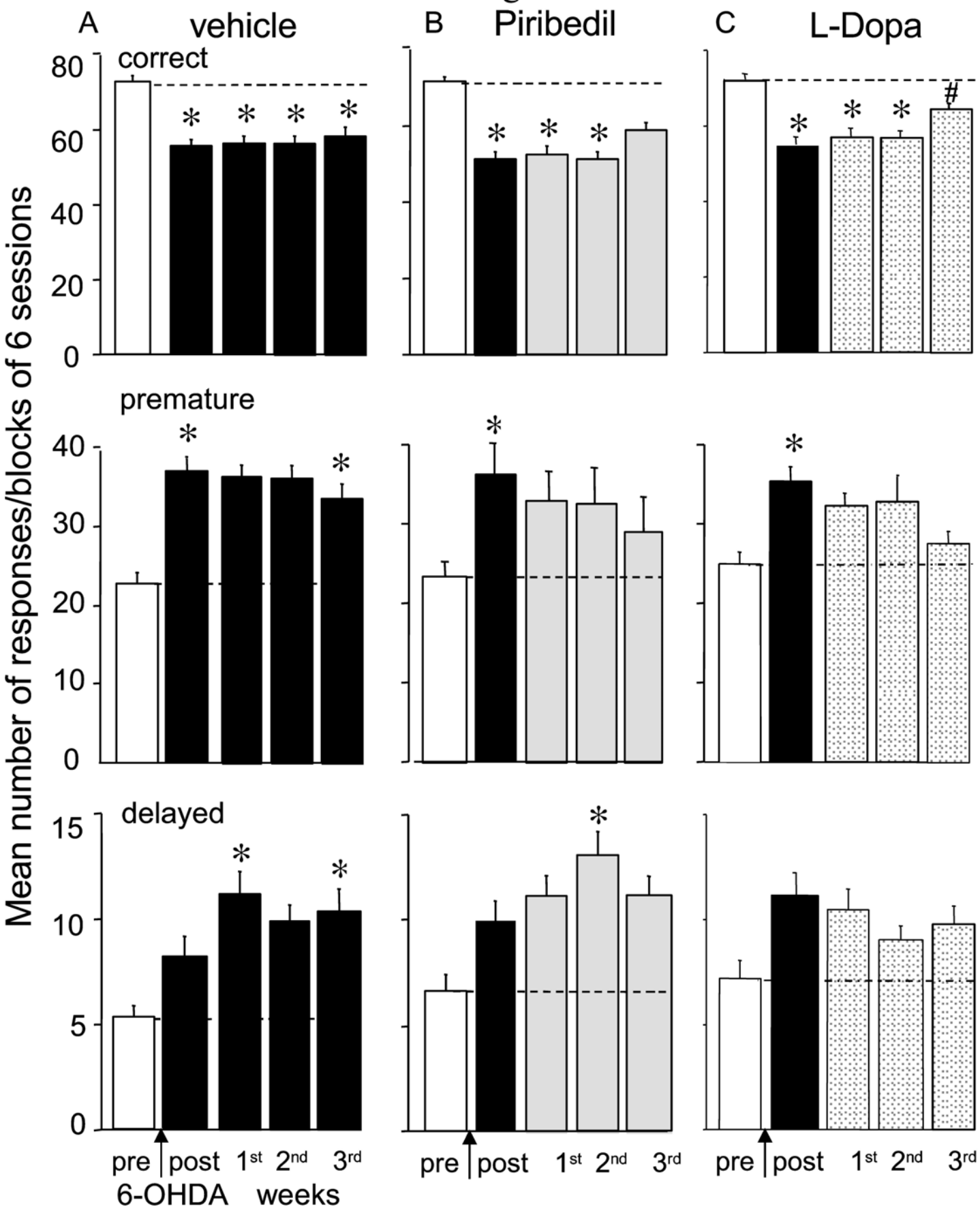

Figure 4

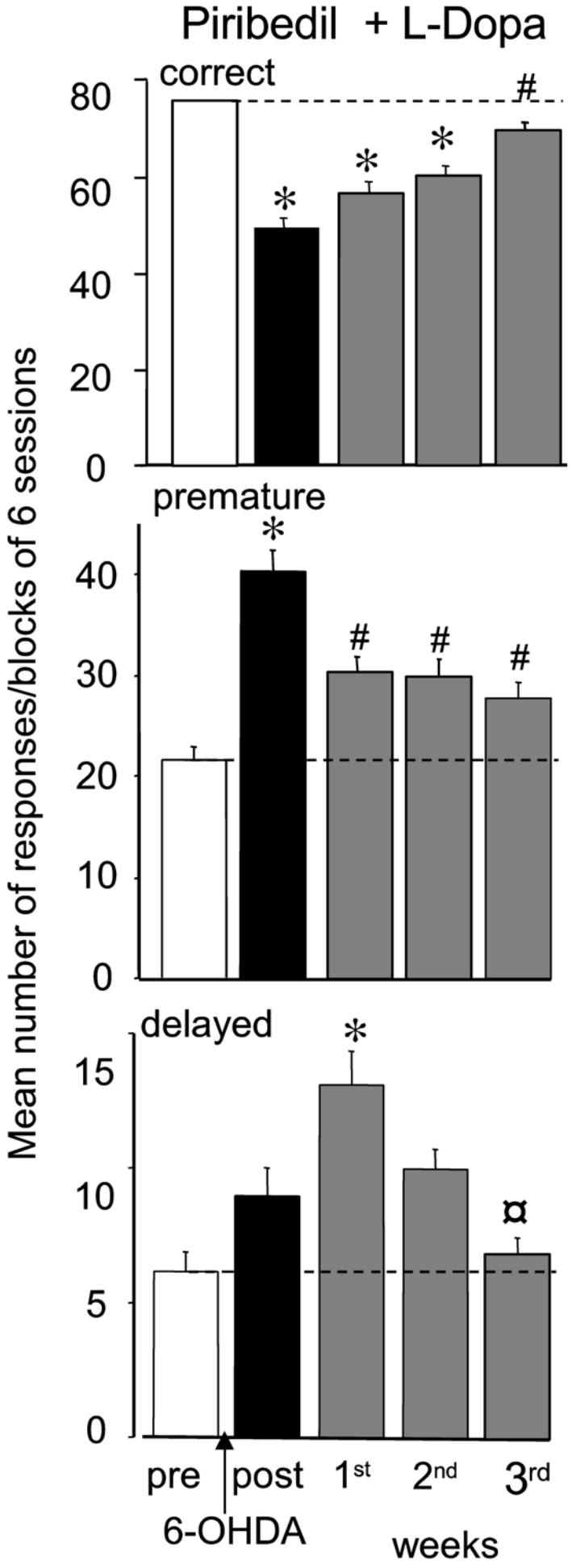

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Figure 5

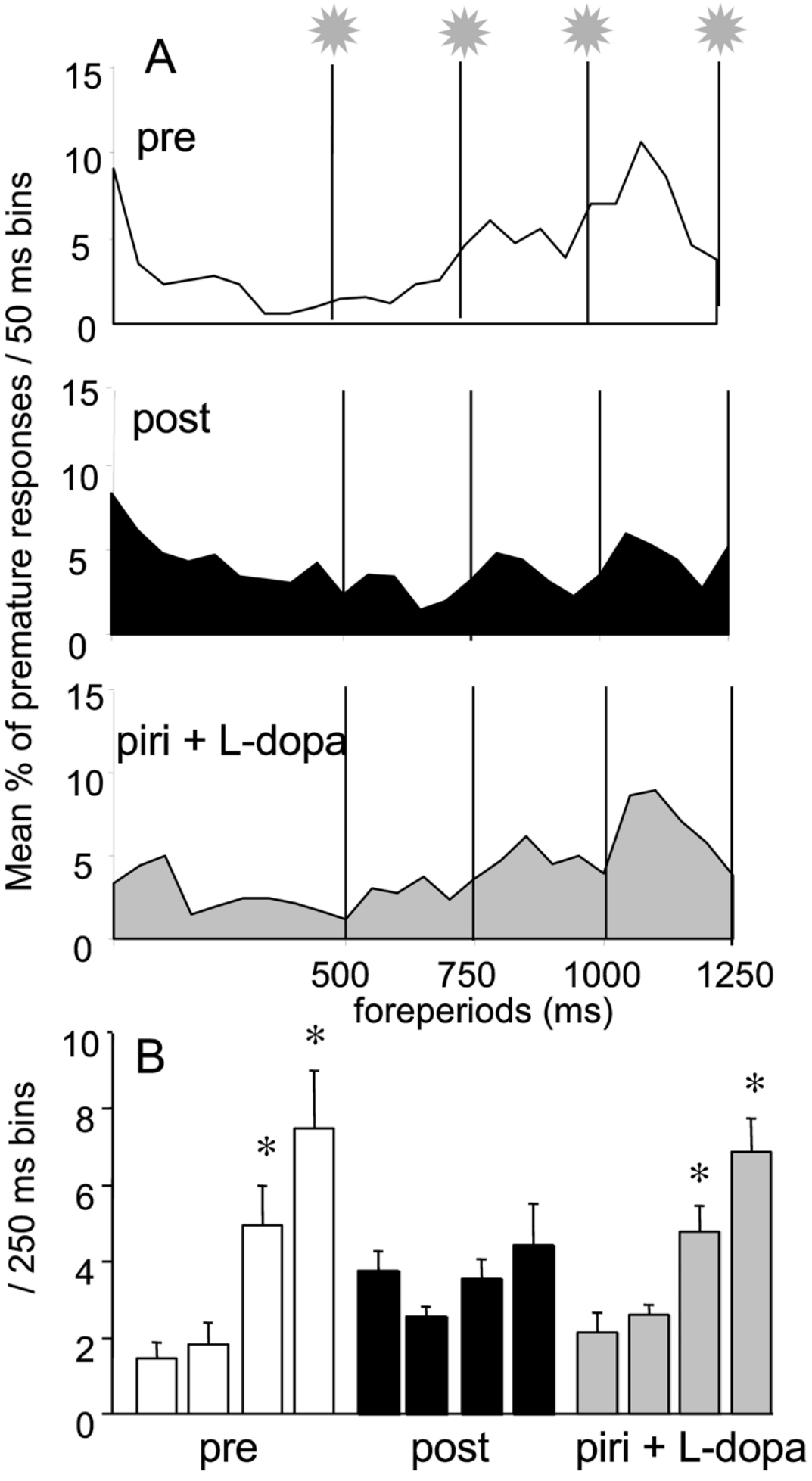

Figure 6

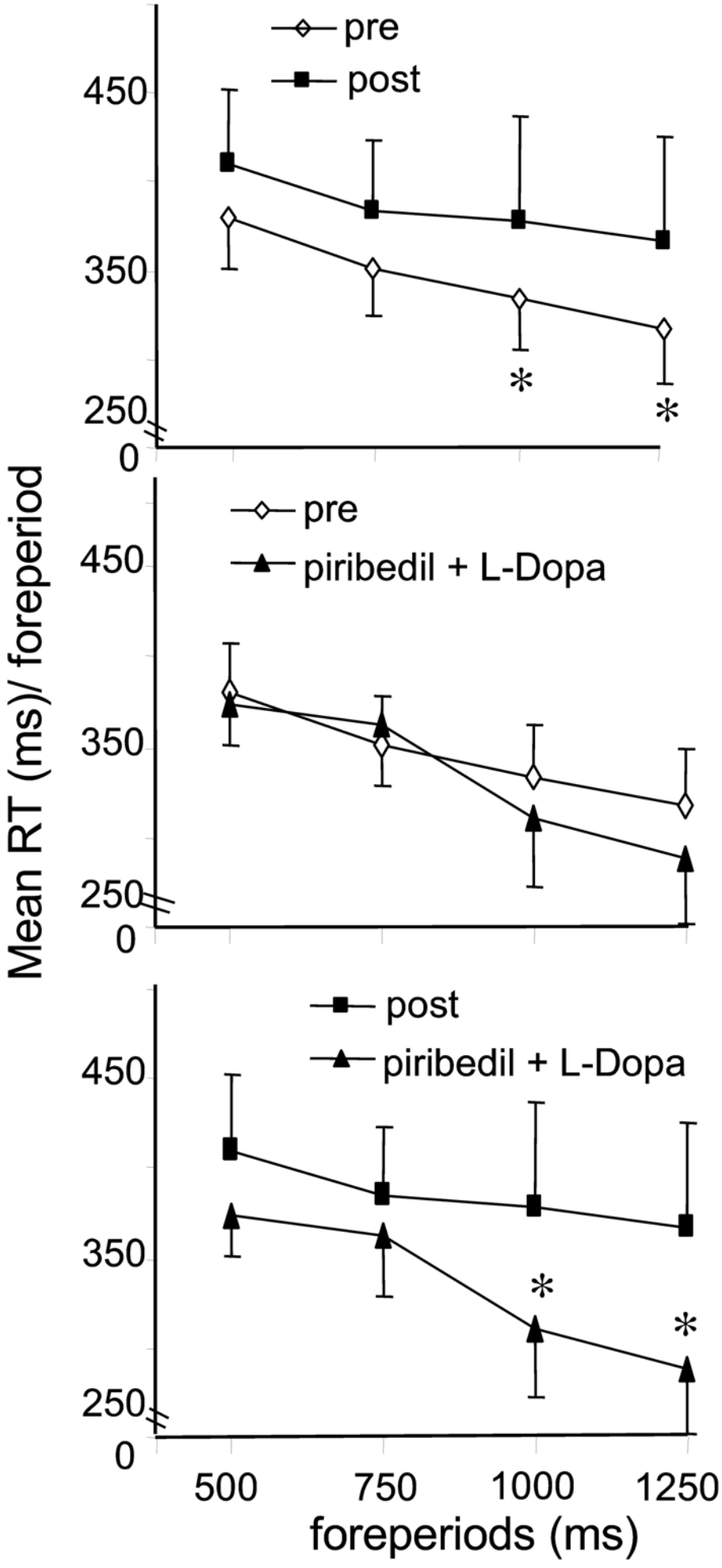

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Figure 7

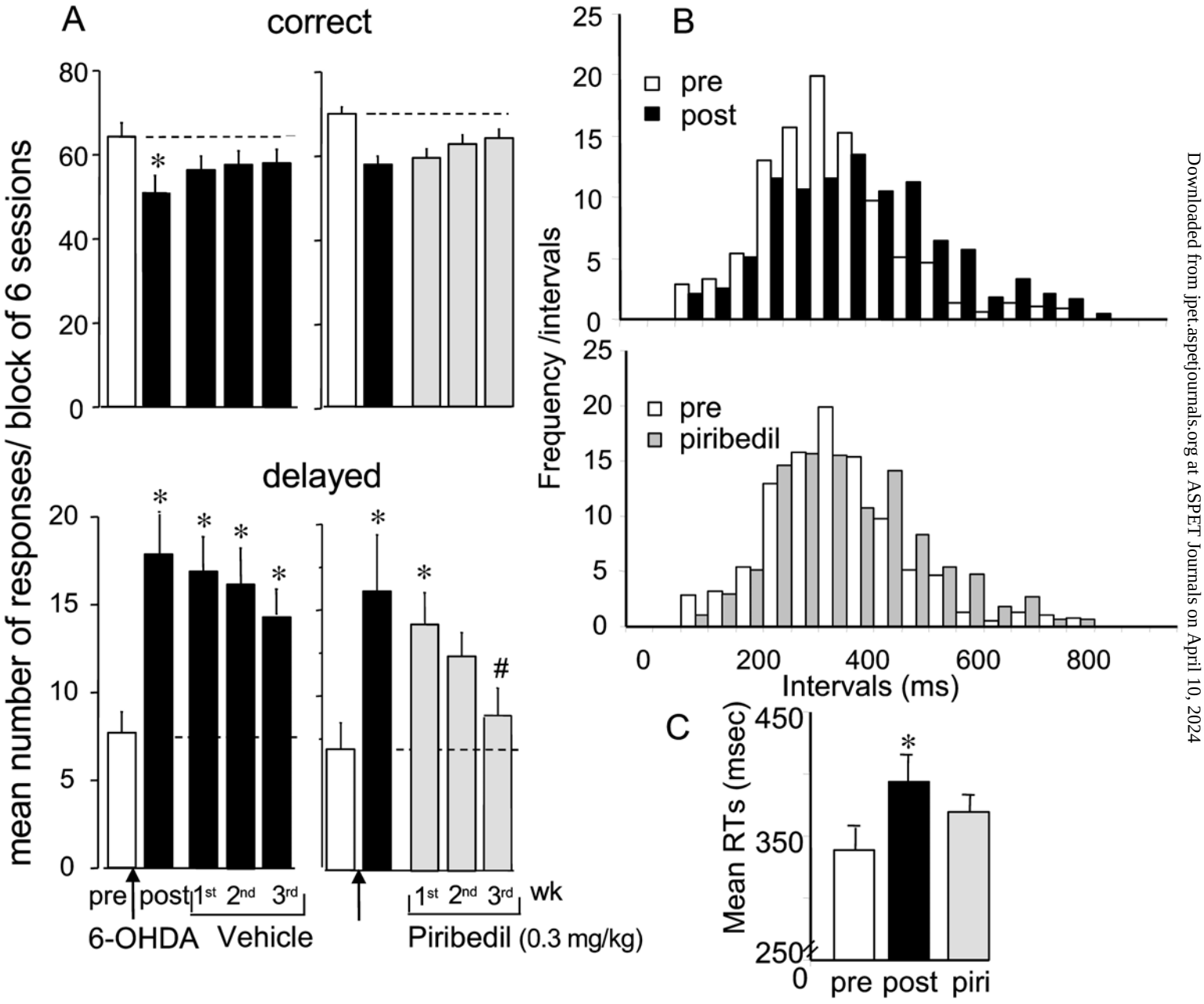

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Figure 8

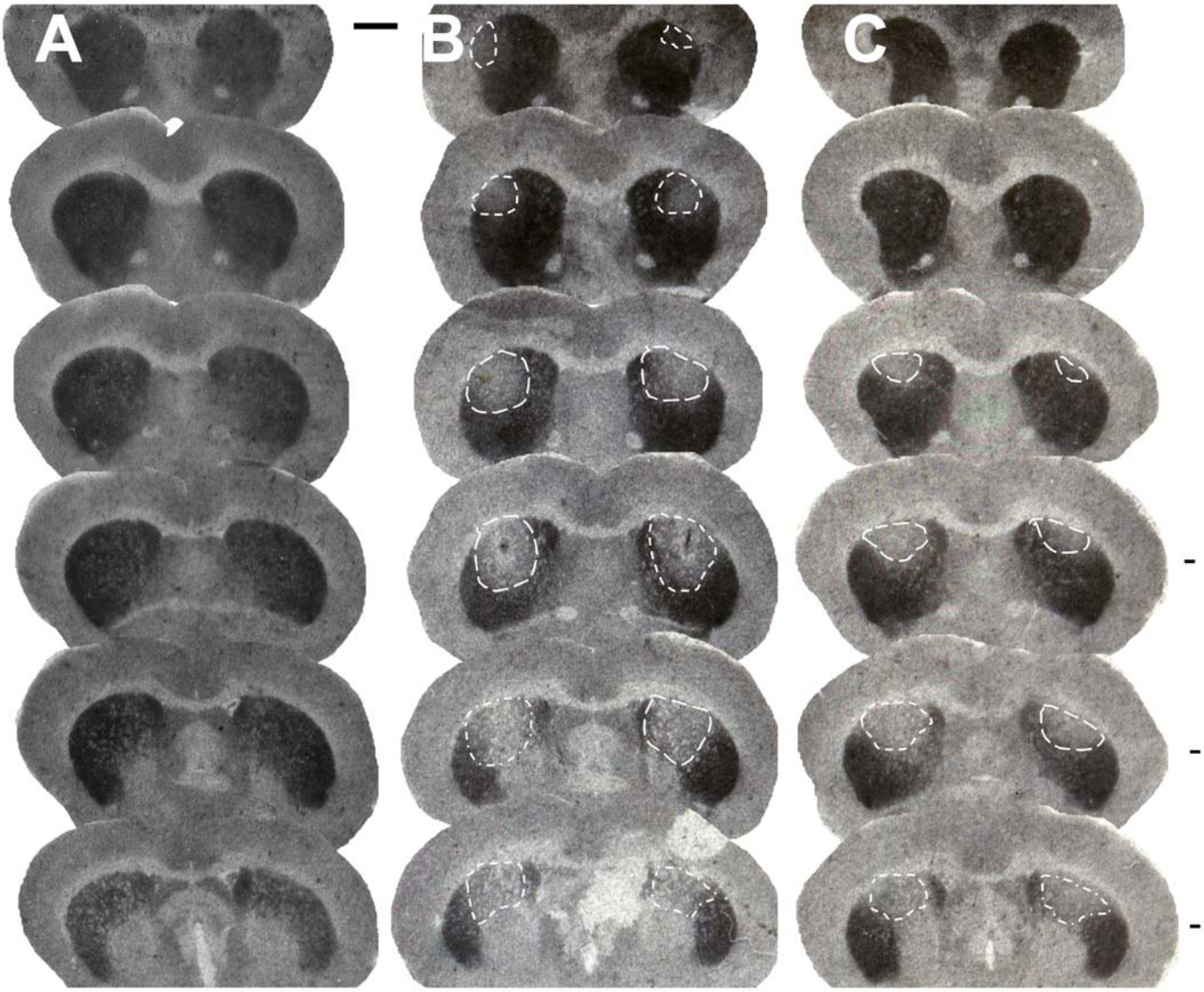

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Figure 9