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Pharmacology, Biochemistry and Behavior 73 (2002) 843–850

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

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Behavioral effects of aminochrome and dopachrome injected in the rat substantia nigra

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Received 14 February 2002; received in revised form 29 May 2002; accepted 6 June 2002

Abstract

The exact mechanism of cell death in neurodegenerative diseases remains obscure, although there is evidence that their pathogenesis may involve the formation of free radicals originating from the oxidative metabolism of catecholamines. The purpose of this study was to evaluate the degree of neurodegenerative changes and behavioral impairments induced by unilateral injection into the rat substantia nigra of cyclized *o*-quinones, aminochrome and dopachrome, derived from oxidizing dopamine and L-DOPA, respectively, with Mn^{3+}–pyrophosphate complex. The behavioral changes were compared with those induced after selective lesions of dopaminergic neurons with 6-hydroxydopamine (6-OHDA). Intranigral injection of aminochrome and dopachrome produced impairment in motor and cognitive behaviors. The behavioral impairment was also revealed by apomorphine-induced rotational asymmetry. Apomorphine (0.5 mg/kg sc) significantly increased rotational behavior in rats injected with aminochrome and dopachrome. These rats presented a clear motor bias showing a significant contralateral rotation activity, similar but less vigorous that in rats injected with 6-OHDA. The avoidance conditioning was seriously impaired in rats injected with aminochrome and dopachrome although only dopachrome-injected rats showed a similar hypomotility to 6-OHDA-injected rats. The behavioral effects were correlated to the extent of striatal tyrosine hydroxylase (TH)-positive fiber loss. Rats receiving unilateral intranigral aminochrome and dopachrome injections exhibited a 47.9 ± 5.1% and a 39.7 ± 4.4% reduction in nigrostriatal TH-positive fiber density. In conclusion, this study provided evidence that oxidizing DA and L-DOPA to cytotoxic quinones, aminochrome and dopachrome appears to be an important mediator of oxidative damage in vivo.

Keywords: Aminochrome; Dopachrome; Dopamine; Neurodegeneration; Parkinsonism

1. Introduction

Although the mechanisms of cell death in neurodegenerative diseases remain unclear, it is accepted that the occurrence of an enhanced oxidative stress may be a common component of degeneration of the nigrostriatal dopaminergic system in diseases like Parkinsonism (Jenner, 1996; Foley and Riederer, 2000). The formation of reactive oxygen species can be extremely dangerous to the brain, due to the great catecholamine concentration and the high speed of oxidative metabolism catalyzed by these metals (Stokes et al., 1999). One possible source of free radicals may involve the reductive metabolism of oxidized forms of dopamine and related catechols (Baez et al., 1995; Segura-Aguilar et al., 1998; Paris et al., 2001). Catecholamines can be oxidized to *o*-quinones by oxygen or transition metal ions like manganese, iron or copper (Archibald and Tyree, 1987; Shen and Dryhurst, 1998; Stokes et al., 1999; Paris et al., 2001). The *o*-quinone generated contributes to redox cycling and can have important consequences in the increment of oxidative stress in neurons due to the formation of highly reactive oxygen species (Dutrait et al., 1995; Lievre et al., 2001; Yoneyama et al., 2001; Paris et al., 2001). Thus, *o*-quinones appear to be important mediators of oxidative damage in vivo and can have a central role in the pathogenesis of several neurodegenerative disorders like Parkinson's disease (Hastings et al., 1996; Berman and Hastings, 1999). The impairment of dopaminergic transmission along

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0091-3057/02/$ – see front matter © 2002 Elsevier Science Inc. All rights reserved.
PII: S0091-3057(02)00923-1

the nigrostriatal pathway has an impact on motor and cognitive behavior (Schneider and Pope-Coleman, 1995; Dubois and Pillon, 1997; Kulisevsky, 2000). Several in vitro reports have demonstrated that the neurotoxic effect of DA and L-DOPA can be linked to their autoxidation to form reactive quinones (Lai and Yu, 1997; Segura-Aguilar et al., 1998; Stokes et al., 1999; Paris et al., 2001). Recently, it was demonstrated that aminochrome resulting from oxidizing of DA with Mn^{3+} was toxic in a mouse-derived neuronal cell line (CNh) (Arriagada et al., 2000) and that Mn^{3+} and dicumarol induce contralateral rotation similar to 6-hydroxydopamine (6-OHDA) (Segura-Aguilar et al., 2002). In order to study the in vivo effects of the reactive *o*-quinones of DA and L-DOPA, we injected aminochrome and dopachrome in the substantia nigra. We evaluated the degeneration of the nigrostriatal pathway through the expression of motor responses (rotational model and spontaneous motor activity) and avoidance conditioning, considering the influence of integrity of DA systems on these behaviors.

2. Methods

2.1. Animals

A total of 32 Sprague–Dawley rats, weighing 180–220 g, were housed six per cage with free access to food and water. They were maintained under a 12:12 light–dark cycle (lights on from 0800 to 2000 h). All the experimental protocols followed the Guide for Care and Use of Laboratory Animals and were approved by the Faculty of Medicine Committee (Protocol CBA#005, FMUCH).

2.2. Stereotaxic injection

Rats were anaesthesized with sodium pentobarbital (30 mg/kg ip) and placed in a David Kopf stereotaxic frame. Stereotaxic coordinates relatives to bregma were AP = − 4.8, ML = − 1.8 and DV = − 8.2, according to Paxinos and Watson (1986). Each solution was injected into the right substantia nigra at a rate of 2 μl/min using a 5-μl Hamilton microsyringe. To minimize the possibility of back flow, the needle remained in position for an additional minute after each injection. Control animals received a corresponding volume of vehicle. After surgery, rats were allowed to recover for 7 days prior to conducting rotational responses to apomorphine (0.5 mg/kg sc). Fourteen days after injection, the rats were submitted to other behavioral experiments by using a fixed design: spontaneous motility followed by conditioned avoidance training.

2.3. Drugs

The following compounds were purchased from Sigma (St. Louis, MO, USA): dopamine, L-DOPA, 6-hydroxydopamine hydrobromide (6-OHDA; 32 nmol). Aminochrome (1.2 nmol) and dopachrome (1.2 nmol) were prepared by oxidizing dopamine and L-DOPA, respectively, with Mn^{3+}–pyrophosphate complex according to Segura-Aguilar and Lind (1989) and Baez et al. (1994), respectively.

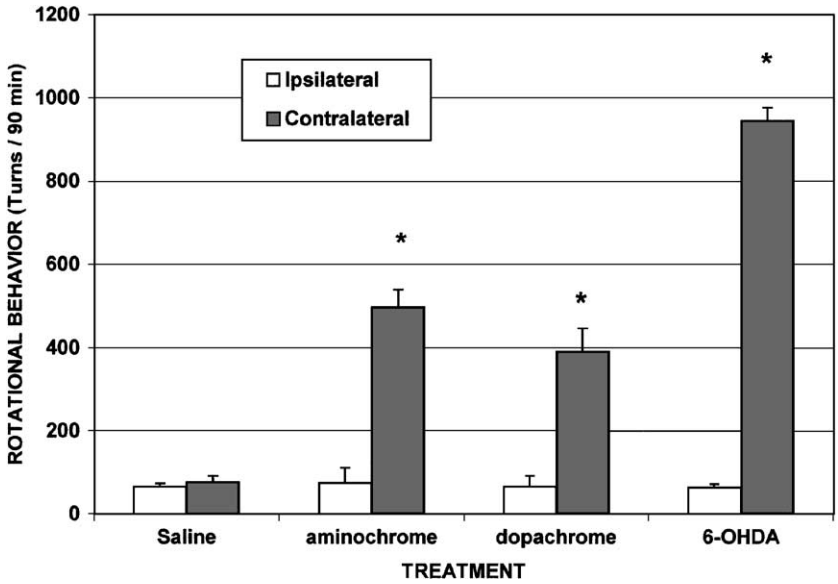

Fig. 1. Rotational behavior induced by apomorphine (0.5 mg/kg sc) in rats with unilateral injection of saline, aminochrome, dopachrome and 6-OHDA into the substantia nigra. The rotational behavior was measured 7 days after intranigral administration (see Methods). The values are the mean ± S.E.M. of eight animals on each group. Bars represent the total ipsilateral and contralateral rotation in a 90-min observation period. For statistical comparisons, we used one-way ANOVA followed by post hoc Newman–Keuls test. * $P < .05$ compared with saline group.

The Mn^{3+}–pyrophosphate complex was prepared according to Archibald and Fridovich (1982).

2.4. Rotational behavior

The rotational model is based on a unilateral lesion of the nigrostriatal DA system with the neurotoxin 6-OHDA (Herrera-Marschitz and Ungerstedt, 1984). Rats with this lesion show a postural deviation, which can be expressed as contralateral rotation behavior when they are stimulated with apomorphine (0.5 mg/kg sc). One week after intranigral drugs injection, the rats were placed in a rotometer to evaluate the presence of motor asymmetry by stimulating the animals with subcutaneous injection of apomorphine (0.5 mg/kg). Apomorphine was dissolved in a solution of 0.02% ascorbic acid in 0.9% saline and was injected in a volume of 1 ml/kg body weight. Rotational behavior testing was carried out in a LE 902 Rotometer (Letica Instruments, Barcelona, Spain), which allowed free movement of the rat for 90 min, connected to a LE 3806 multicounter (Letica Instruments). The sensor measured and provided a separate output for continuous movement in the clockwise or counterclockwise direction. Results were expressed as the mean total number of complete 360° turns in either direction during the entire period of observation.

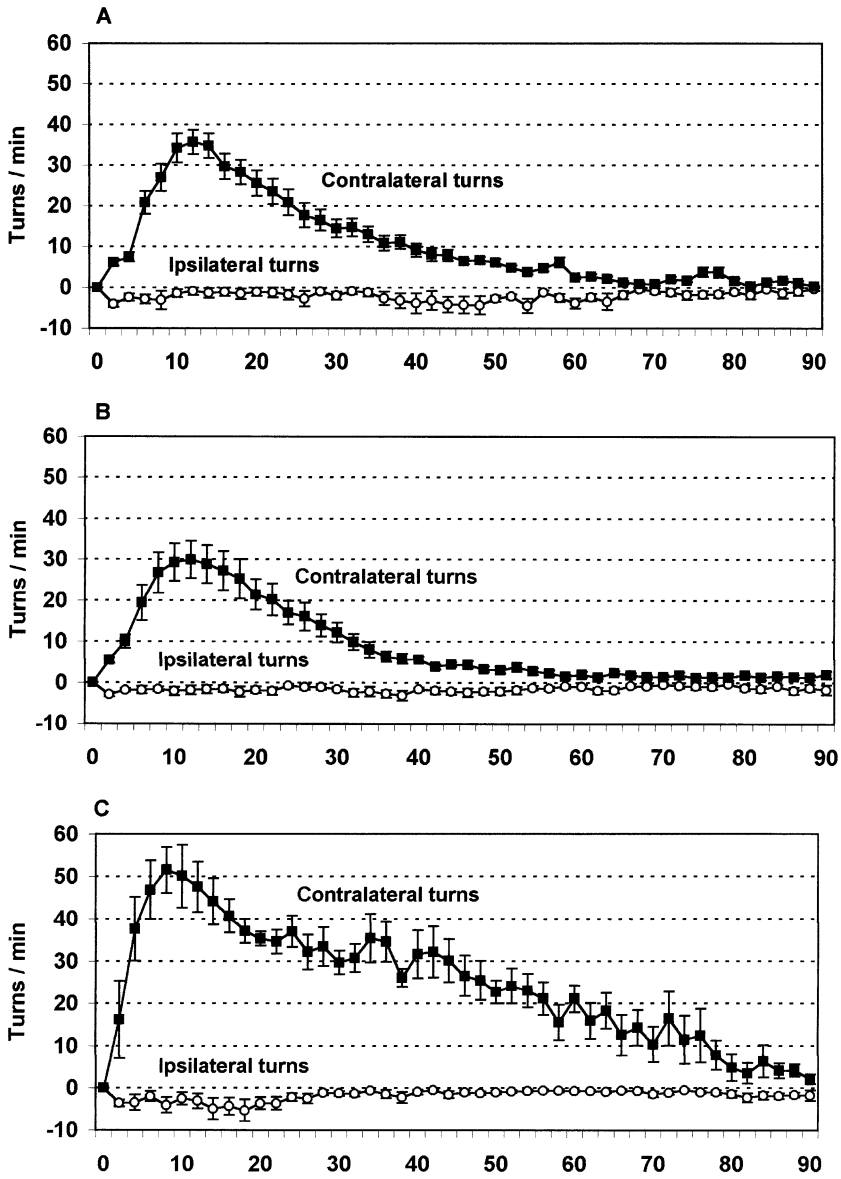

Fig. 2. Rotational patterns induced by apomorphine (0.5 mg/kg sc) in rats with unilateral injection of aminochrome, dopachrome and 6-OHDA into the substantia nigra. The rotational behavior was measured 7 days after intranigral administration (see Methods). Each curve represents contralateral and ipsilateral rotational patterns produced by aminochrome (A), dopachrome (B) and 6-OHDA (C) injection expressed as turns per minute.

2.5. Spontaneous motor activity

Fourteen days after stereotaxic injection, each rat was individually placed in a Plexiglas cage ($30 \times 30 \times 30$ cm) inside a soundproof room. The floor of the cage was an activity platform (Lafayette Instrument, USA) connected to an electromechanical counter. Spontaneous motor activity was monitored for 15 min.

2.6. Active avoidance conditioning

Immediately after the spontaneous motor activity test, each rat was individually placed in a two-way shuttle box (Lafayette Instrument) composed of two stainless steel modular testing units. Each unit was equipped with an 18-bar insulated shock grid floor, two 28-V DC lights and a tone generator (Mallory Sonalert 2800 Hz; Lafayette Instrument). Electric shocks were provided to the grid floor by a master shock supply (Lafayette Instrument). The rats were trained over 50 trials, after a 5-min period of habituation. The trial consisted of the presentation of a tone that after 5 s was overlapped with a 0.20-mA footshock until the animal escaped to the opposite chamber, with maximum shock duration of 10 s. A conditioned avoidance response (CAR) was defined as a crossing to the opposite chamber within the first 5 s (tone alone).

2.7. Immunohistochemistry

After completing behavioral testing, the rats were administrated an overdose of sodium pentobarbital and perfused intracardially with 0.9% saline followed by 4% buffered formaldehyde solution. The brains were removed, fixed in a 4% buffered formaldehyde solution for 24 h and cryoprotected in 30% sucrose. All samples were cut into 10-μm-thick sections on a cryostat. Coronal sections at forebrain medial bundle level were processed for immunohistochemical demonstration of tyrosine hydroxylase (TH) and for evaluation of the consequences of intranigral injections of aminochrome and dopachrome. Immunohistochemistry was performed with the ExtrAvidin Peroxidase Staining kits (Sigma). The degree of DA nigrostriatal lesion was expressed as the percentage of reduction in TH-positive fiber density in the injected side as compared to the contralateral noninjected side. The quantification of TH-positive fiber density was made by counting the total of fibers in a transversal section of the medial bundle, using a computer image analysis system consisting of an Olympus BH-2 microscope equipped with a DEGUS video camera coupled to a Power Mac G4 computer with the NIH image analysis software.

2.8. Statistical analysis

Results were expressed as mean ± S.E.M. The data were analyzed statistically by one-way analysis of variance (ANOVA), followed by post hoc Newman–Keuls' multiple comparison tests, when appropriate. The level of statistical significance was set at $P < .05$.

3. Results

3.1. Rotational behavior

Apomorphine (0.5 mg/kg sc) produced rotational behavior in rats injected with aminochrome, dopachrome and 6-OHDA (Fig. 1). The one-way analysis of variance (ANOVA) shows that these rats presented a clear motor bias, rotating towards the contralateral side to the intranigral injection [$F(3,28) = 11.41$, $P < .0001$]. Post hoc analysis indicated that the 6-OHDA-lesioned rats showed a strong

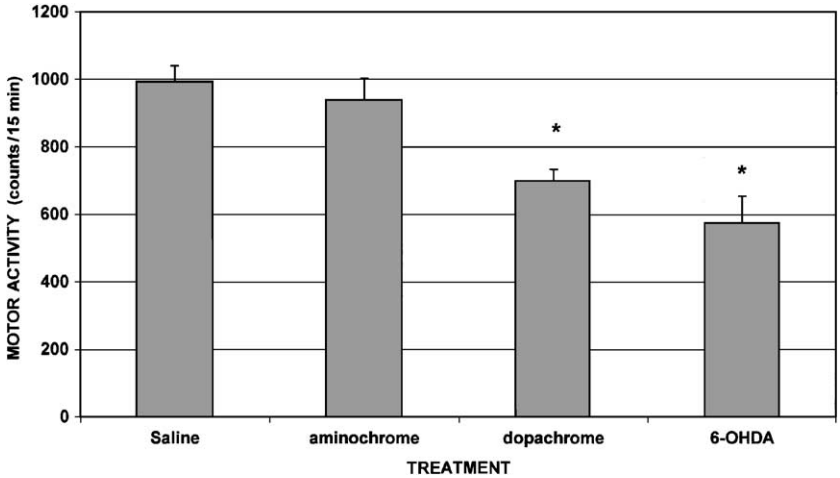

Fig. 3. Spontaneous motor activity in rats with unilateral injection of saline, aminochrome, dopachrome and 6-OHDA into the substantia nigra. This behavior was measured 14 days after intranigral administration (see Methods). The values are the mean ± S.E.M. of eight animals on each group. Bars represent the total spontaneous motor activity in a 15-min observation period. For statistical comparisons, we used one-way ANOVA followed by post hoc Newman–Keuls test. * $P < .05$ compared with saline group.

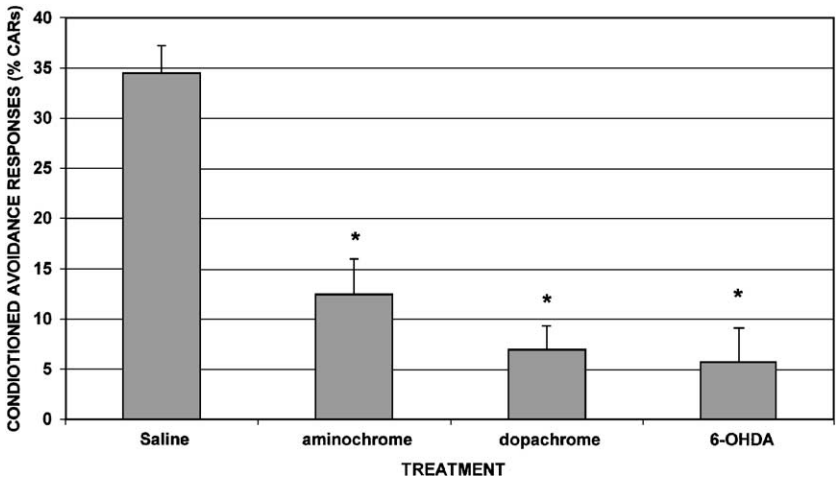

Fig. 4. Conditioned avoidance responses in rats with unilateral injection of saline, aminochrome, dopachrome and 6-OHDA into the substantia nigra. This behavior was measured 14 days after intranigral administration (see Methods). The values are the mean ± S.E.M. of eight animals on each group. Bars represent the percentages of conditioned avoidance responses for 50 trials. For statistical comparisons, we used one-way ANOVA followed by post hoc Newman–Keuls test. * $P < .05$ compared with saline group.

and characteristic contralateral rotational pattern, as previously described (Herrera-Marschitz and Ungerstedt, 1984), by stimulating striatal DA receptors rendered supersensitive in the denervated side. These animals rotated a total of 945 ± 85 contralateral turns in the 90-min period of observation. This behavior was significantly more pronounced in 6-OHDA-injected rats than in all the other groups of rats. However, systemically, apomorphine injection produced a significant contralateral rotation in rats with intranigral injection of aminochrome and dopachrome. Rats injected with aminochrome and dopachrome rotated in a similar way, 498 ± 55 and 391 ± 45 contralateral turns, respectively. Rats injected with saline did not show any significant rotational response. Fig. 2 shows rotational pattern, expressed as rotation intensity (turns/min), induced by apomorphine in rats with unilateral injection of aminochrome, dopachrome and 6-OHDA into the substantia nigra. In aminochrome-and dopachrome-injected groups, the rotational intensity reach a peak 10–20 min after the apomorphine injection. Injections of 6-OHDA induced a contralateral response with increase in intensity and duration (Fig. 2).

3.2. Spontaneous motor activity

In Fig. 3, we observed the effects of intranigral injections on spontaneous motor activity. One-way ANOVA revealed a significant effect of the treatment [$F(3,28) = 10.93$, $P < .0001$] on motility. Subsequent Newman–Keuls' test indicated that only dopachrome-injected rats show evident hypomotility similar to 6-OHDA-injected rats.

3.3. Active avoidance conditioning

The results of the active avoidance conditioning are showed in Fig. 4. One-way ANOVA revealed a significant effect of the stereotaxic injection [$F(3,28) = 14.59$, $P < .0001$] on the acquisition of CARs. Post hoc comparisons indicated that the avoidance conditioning was seriously impaired in rats injected with aminochrome and dopachrome. These rats showed a similar response that observed in rats injected with 6-OHDA.

3.4. Immunohistochemistry

Unilateral injection of aminochrome and dopachrome results in a significant loss of tyrosine hydroxylase immunoreactivity within the nigrostriatal pathway ($P < .05$). Rats receiving unilateral intranigral aminochrome and dopachrome injections exhibited a 47.9 ± 5.1% and a 39.7 ± 4.4% reduction in nigrostriatal TH-positive fiber density compared to the contralateral noninjected side. The reduction of tyrosine hydroxylase immunoreactivity in control rats receiving unilateral injection of 6-OHDA was 84.2 ± 3.1%.

4. Discussion

The present study was concerned with investigating the behavioral effects of aminochrome and dopachrome injected unilaterally into the substantia nigra. These effects were compared with those induced after selective lesion of dopaminergic neurons with unilateral intranigral injection of 6-OHDA. This neurotoxin has been used extensively in animal model of Parkinson's disease (Flint Beal, 2001). A stereotaxic unilateral intranigral injection of 6-OHDA produces a well-localized degeneration at the nigrostriatal DA system. Ungerstedt et al. (1974) demonstrated that following unilateral nigrostriatal DA denervation with 6-OHDA, the rats developed a postural deviation. This postural deviation could be transformed into strong rota-

tional behavior towards the opposite side to the lesion (contralateral rotation) by stimulating the rats with apomorphine at least 1 week after 6-OHDA injection. This effect of apomorphine is due to stimulation of supersensitive DA receptors located in the denervated side (Marshall and Ungerstedt, 1977). In the present study, only intranigral injection of dopachrome induced a significant decrease of spontaneous global motor activity similar to 6-OHDA; however, the injection of both *o*-quinones affected the rotational behavior. Aminochrome and dopachrome intranigral injection elicited a significant contralateral response, suggesting a selective denervation of the nigrostriatal DA system. In these rats, we also observed an inhibitory effect on the acquisition of an avoidance task. Two-way avoidance is a type of conditioning that results in associative learning. The rat learns to avoid a signaled noxious stimulus (electrical shock) by initiating a locomotor response for moving to another compartment. Although acquisition of avoidance responses could be altered by changes in locomotor activity, the present behavioral data suggest that the influences of both *o*-quinones on avoidance response were not necessarily consequence of equivalent changes in spontaneous motor activity. In 6-OHDA intranigral-injected rats, depressed motor activity was clearly accompanied by a decrease in avoidance conditioning. Similar effects were shown by dopachrome injection, with a lesser effect on motility along with an important decrease in conditioning. With the aminochrome injection, both behaviors were dissociated. In fact, the impairment in avoidance acquisition was not accompanied by any change in motor activity. Therefore, in our experimental conditions, the cognitive functions seem to be more sensitive than the spontaneous motor performance to the neurotoxic effect of *o*-quinones. Although dopamine systems have been implicated in the performance of avoidance behavior, there are a few studies about the effects of dopamine depletion by 6-OHDA on active avoidance in rats. All these behavioral changes correlated well with significant loss of tyrosine hydroxylase (TH) immunoreactive at medial forebrain bundle, confirming the neurotoxicity of both *o*-quinones.

Traditionally, the role of basal ganglia in the control of movement has been stressed, but experimental work has shown that cognitive behavioral deficits are also associated with striatum damage. Some data concerning basal ganglia output not only underscore their involvement in cognitive processes but also suggest that the nature of the involvement is at least in part related to working memory and perception (Middleton and Strick, 1996). Later studies of idiopathic Parkinson's disease may involve not only a disruption in the basal ganglia, but also a disruption in the frontal cortex, in which dopamine may be depleted as well. Nevertheless, behavioral deficits have been seen at very early stages of Parkinsonism when dopamine depletion is primarily in the nigrostriatum system and not in the frontal cortex (Dubois and Pillon, 1997). Our results support the suggestion of Schneider and Pope-Coleman (1995) that the cognitive impairment in Parkinson's disease may precede the motor signs of the disease and not caused by them. In fact, following chronic low-dose MPTP exposure, monkeys develop cognitive impairments without gross motor impairments (Schneider, 1990; Schneider and Kovelowski, 1990). Neurochemical analysis of the brain of these animals has shown a primary striatal dopaminergic deficit (Schneider, 1990) with no significant cortical dopaminergic deficit, suggesting that the cognitive dysfunction is centered in the striatum rather in the frontal cortex.

Our results support the suggestion that the neurodegenerative events in dopaminergic systems depend on overproduction of aminochrome and dopachrome. The questions are: Can they be produced in vivo and what is their role in the degenerative processes of dopaminergic systems observed in Parkinson's disease? The existence in vivo of *o*-quinones is supported by: (i) the finding that cysteinyl adducts, such as 5-cysteinyldopamine, 5-cysteinyldopa and quinone adducts, are formed during oxidative metabolism of dopamine/DOPA in rat, guinea pig and human brain (Rosengren et al., 1985; Carlsson and Fornstedt, 1991; LaVoie and Hastings, 1999). Recently, it has been reported that glutathione transferase (GST) M2-2, expressed in human substantia nigra (Baez et al., 1997), catalyzes glutathione conjugation of dopamine and DOPA *o*-quinone to 5-glutathionyldopamine and 5-glutathionyldopa, which are the precursors of 5-cysteinyldopamine and 5-cysteinyldopa, respectively (Dagnino-Subiabre et al., 2000); and (ii) the presence of neuromelanin in substantia nigra is also an evidence for oxidation of dopamine to *o*-quinones since aminochrome and dopachrome are precursors of this polymer (Costa et al., 1992). Neuromelanin biosynthesis was found to be driven by excess cytosolic catecholamines not accumulated by synaptic vesicles (Sulzer et al., 2000). The low pH in the vesicles prevents dopamine autoxidation due to strong protonation of the catechol group. The autoxidation of dopamine to aminochrome and polymerization to neuromelanin has been postulated to be a normal process since DT-diaphorase and GST M2-2 prevent one-electron reduction of aminochrome to leukoaminochrome *o*-semiquinone radical (Baez et al., 1995; Segura-Aguilar et al., 1997, 1998; Paris et al., 2001). DT-diaphorase has been proposed as an antioxidant and neuroprotective enzyme in Parkinson's disease (Baez et al., 1994; Segura-Aguilar and Lind, 1989). This enzyme is present in the dopaminergic neurons in the substance nigra and constitutes 98% of the total quinines reductase activity (Schultzberg et al., 1988). Leukoaminochrome *o*-semiquinone radical has been reported to be responsible for neurotoxic effects of aminochrome in dopaminergic RCSN-3 cells derived from rat substantia nigra when DT-diaphorase was inhibited by dicumarol (Paris et al., 2001). Intracerebral Mn^{3+} administration, together with the DT-diaphorase inhibitor dicumarol in the left medial forebrain bundle, produced a behavioral pattern characterized by contralateral behavior when the rats

were stimulated with apomorphine, in a manner similar to that when administered to unilaterally 6-OHDA-lesioned animals (Segura-Aguilar et al., 2002).

Our behavioral results are in agreement with studies that confirm the aminochrome and dopachrome toxicity in vitro (Lai and Yu, 1997; Arriagada et al., 2000; Paris et al., 2001). They also led us to suggest a role of oxidative metabolism of dopamine and L-DOPA in the severe side effects observed in Parkinson's disease patients treated with L-DOPA.

Acknowledgements

This work was supported by grant 1990622 from Fondecyt, Chile.

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