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RESEARCH ARTICLE

Jordi Bové · Jordi Serrats · Guadalupe Mengod
Roser Cortés · Eduardo Tolosa · Concepció Marin

Neuroprotection induced by the adenosine A_{2A} antagonist CSC in the 6-OHDA rat model of parkinsonism: effect on the activity of striatal output pathways

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Abstract In Parkinson's disease (PD), the striatal dopamine depletion and the following overactivation of the indirect pathway of the basal ganglia leads to very early disinhibition of the subthalamic nucleus (STN) that may contribute to the progression of PD by glutamatergic overstimulation of the dopaminergic neurons in the substantia nigra. Adenosine A_{2A} antagonism has been demonstrated to attenuate the overactivity of the striatopallidal pathway. To investigate whether neuroprotection exerted by the A_{2A} antagonist 8-(3-chlorostyryl)caffeine (CSC) correlates with a diminution of the striatopallidal pathway activity, we have examined the changes in the mRNA encoding for enkephalin, dynorphin, and adenosine A_{2A} receptors by in situ hybridization induced by subacute systemic pretreatment with CSC in rats with striatal 6-hydroxydopamine(6-OHDA) administration. Animals received CSC for 7 days until 30 min before 6-OHDA intrastriatal administration. Vehicle-treated group received a solution of dimethyl sulfoxide. CSC pretreatment partially attenuated the decrease in nigral tyrosine hydroxylase immunoreactivity induced by 6-OHDA, whereas no modification of the increase in preproenkephalin mRNA expression in the dorsolateral striatum was observed. The neuroprotective effect of the adenosine A_{2A} antagonist CSC in striatal 6-OHDA-lesioned rats does not result from a normalization of the increase in striatal PPE mRNA expression in the DL striatum, suggesting that other different mechanisms may be involved.

Keywords Parkinson · Adenosine A_{2A} receptors · Enkephalin · Dynorphin

J. Bové · E. Tolosa · C. Marin (✉)
Laboratori de Neurologia Experimental, Área de Neurociències, Fundació Clinic-Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Villarroel 170, 08036 Barcelona, Spain
E-mail: cmarin@clinic.ub.es
Tel.: +34-93-2275400
Fax: +34-93-4515272

J. Serrats · G. Mengod · R. Cortés
Departament de Neuroquímica, Institut d'Investigacions Biomèdiques de Barcelona, CSIC-IDIBAPS Barcelona, Spain

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that progresses over years affecting prominently the dopaminergic neurons of the substantia nigra pars compacta (SNc). Indeed, most of the disabling motor symptoms of PD are due to this neuronal loss and the concomitant dramatic reduction of the dopamine content in the striatum. Although several dopaminomimetic drugs are useful in relieving motor symptoms, none of them clearly diminishes or prevents the progression of the disease.

Recently, adenosine A_{2A} receptor antagonists have appeared to have an anti-parkinsonian effect in several experimental models of PD (Kanda et al. 1998, 2000; Grondin et al. 1999; Koga et al. 2000; Pinna et al. 2001), and to reverse levodopa-induced motor fluctuations (Bové et al. 2002). Adenosine A_{2A} receptors are mainly expressed in the striatum (Jarvis and Williams 1989; Ongini and Fredholm 1996; Moreau and Huber 1999; Svenningsson et al. 1999; Kaelin-Lang et al. 2000; El Yacoubi et al. 2001) and colocalized with preproenkephalin mRNA (Schiffmann et al. 1991; Augood and Emson 1994; Augood 1999) and dopamine (DA) D-2 receptor mRNA (Fink et al. 1992; Pollack et al. 1993; Johansson et al. 1997) in the striatopallidal medium spiny neurons that constitute the so-called indirect pathway of the basal ganglia. In PD (Miller and DeLong 1987; Bergman et al. 1990) and in experimental models (Mitchell et al. 1989; DeLong 1990), it has been demonstrated that this output pathway is overactive. This

phenomenon is revealed by up-regulation of enkephalin and its encoding mRNA in humans (Grafe et al. 1985; Nisbet et al. 1995; Calon et al. 2002), monkeys (Asselin et al. 1994; Herrero et al. 1995; Jolkkonen et al. 1995; Morissete et al. 1997) and rats (Voorn et al. 1987; Gerfen et al. 1990; Jian et al. 1990; Engber et al. 1991; Nisenbaum et al. 1994; Carta et al. 2002). A deficient level of striatal dopamine and the following overactivation of the indirect pathway leads to a very early (Vila et al. 2000) disinhibition of the subthalamic nucleus (STN) and, subsequently, to excessive subthalamopallidal drive. This results in decreased facilitation of cortical motor areas and consequent development of akinesia and bradykinesia (Bergman et al. 1990).

In addition to its main targets, the STN also sends excitatory projections to the dopaminergic neurons in the SNc (Kita and Kitai 1987). Therefore, it has been postulated that the subthalamic desinhibition may also contribute to the progression of PD by glutamatergic overstimulation of SNc neurons, leading to a vicious circle in which STN overactivity and nigral damage support each other (Rodriguez et al. 1998). In agreement with this notion, it has been shown that reducing STN activity by means of local infusion of the glutamate antagonist MK801 (Blandini et al. 2001) or STN lesion (Piallat et al. 1996, 1999) protects SNc neurons from 6-OHDA neurotoxicity.

Adenosine A_{2A} antagonism has been demonstrated to attenuate the overactivity of the striatopallidal pathway since systemic administration of adenosine A_{2A} receptor antagonists reverses increased gammaminobutyric acid (GABA) release in the globus pallidus (Ochi et al. 2000) and reverses the increased expression of preproenkephalin (PPE) in the striatum of unilateral 6-OHDA-lesioned rats (Aoyama et al. 2002). On the basis of these data, it seems reasonable that adenosine A_{2A} antagonists might exert a neuroprotective effect, at least in part, by counteracting striatopallidal pathway overactivity, and therefore reducing the glutamatergic input to the SNc from the STN.

With regard to neuroprotective activity, the A_{2A} antagonists have shown to protect against neuronal damage in excitotoxicity (Jones et al. 1998a, b) and ischemic models (Von Lubitz et al. 1995; Bona et al. 1997; Monopoli et al. 1998). Recent experimental data, has also indicated that A_{2A} antagonists have neuroprotective properties in PD models, specifically in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice and 6-OHDA-treated rats (Chen et al. 2001, 2002; Ikeda et al. 2002; Schwarzschild et al. 2002). In vitro studies have pointed out some possible mechanisms of its neuroprotective effect. On one hand, the A_{2A} antagonist KW-6002 modifies the packaging of [3H]MPP+ into synaptic vesicles (Ikeda et al. 2002) and in the other hand, another A_{2A} antagonist, 8-(3-chlorostyryl)caffeine (CSC), inhibits monoamine oxidase-B (MAO-B) (Chen et al. 2002), although it is still not clear how A_{2A} antagonists exert their neuroprotective effect in PD experimental models.

The aim of the present study was to investigate whether neuroprotection exerted by CSC administration correlates with a diminution of the striatopallidal pathway activity. For this purpose, we examined the striatal changes in the mRNA encoding for enkephalin, dynorphin, and adenosine A_{2A} receptors induced by subacute pretreatment with a selective A_{2A} antagonist CSC (Moreau and Huber 1999) in an experimental model of PD in rats with striatal 6-OHDA administration.

Materials and methods

Animals and protocol treatments

Male Sprague-Dawley rats weighting 240–280 g and housed on a 12-h light/dark cycle with free access to food and water were used for the experiments. Animals received subacute administration of the selective A_{2A} antagonist of 8-(3-chlorostyryl)caffeine (CSC, 5 mg/kg/day, ip, distributed in two injections, $n=7$; Sigma-Aldrich Co., Spain) for 7 days until 30 min before 6-OHDA intrastriatal administration. Vehicle-treated group received a solution of 2% DMSO ip ($n=9$). The dose of CSC used in the present study has been shown to potentiate levodopa effects in several behavioral paradigms (Bové et al. 2002). Animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the local Government.

Striatal 6-OHDA lesion

Animals were anaesthetized with sodium pentobarbital (50 mg/kg, ip) and placed in a stereotaxic frame with the incisor bar positioned at 0 for all injections. Unilateral stereotaxic injections of 6-OHDA (Sigma-Aldrich) were made into the left striatum using a Hamilton syringe. A concentration of 3.0 μg/μl of 6-OHDA hydrobromide dissolved in vehicle was injected into four striatal sites (2 μl/site, total dose 24 μg, $n=16$) at the following coordinates: (1) A: +1.4, L: +2.6, V: −5.0; (2) A: +0.4, L: +3.0, V: −5.0; (3) A: −0.4, L: +4.2, V: −5.0; (4) A: −1.3; L: +4.5, V: −5.0. These coordinates were calculated from bregma and according to the atlas of Paxinos and Watson (1982). Rate of injection was 1 μl/min, leaving the needle in place for a further 2 min before withdrawal. Rats were kept housed as before the experiment for 21 days allowing the progressive degeneration of the nigrostriatal system (Przedborski et al. 1995; Kirik et al. 1998). Sham-lesioned group received intrastriatal administration of 0.2% ascorbic acid/saline ($n=5$).

Rotational behavior test

Rotational behavior induced by 0.5 mg/kg, sc apomorphine (Sigma-Aldrich) administration was measured

21 days after striatal 6-OHDA microinjections. Rats were placed in circular cages and tethered to an automated rotometer. The number of complete (360°) turns made during each 5-min period was recorded by computer. Rats were allowed 15 min to habituate to the rotometer before the administration of apomorphine. The total time of testing after apomorphine administration was of 1 h. Total rotational activity was measured by counting the total number of net contralateral turns after the deduction of the ipsilateral rotations.

Tissue collection

The day after apomorphine administration, rats were killed with an overdose of anesthesia. Brains were quickly removed from the skull and then frozen on dry ice and kept at −80°C until were cut on a cryostat (Leica, Spain). Coronal 14-μm thick sections were collected through the striatum and the substantia nigra pars compacta onto APTS (3-amino-propyltriethoxysilane; Sigma-Aldrich) coated slides, and kept at −40°C until used.

Tyrosine hydroxylase immunohistochemistry

Nigral and striatal sections were defrozed and dried at room temperature and fixed with acetone for 10 min at 4°C. Then were rinsed in phosphate buffered saline (PBS) pH 7.4; Sigma-Aldrich) twice, 5 min each, and immersed in 0.3% hydrogen peroxide (Merck-Schuchardt, Hohennbrunn, Germany) in PBS for 10 min to block the endogenous peroxidase. At this point, sections were rinsed again in PBS and incubated with horse serum (GibcoBRL, Life Technologies Ltd, Auckland, New Zealand) with 0.1% Triton X-100 (Sigma-Aldrich) for 20 min. Sections were incubated overnight at 4°C with mouse anti-tyrosine hydroxylase (TH) monoclonal antibody (Chemicon Int. Inc., Calif., USA) at a dilution 1:500 in PBS. Sections were rinsed twice in PBS, 5 min each, and ImmunoPure Ultra-Sensitive ABC Peroxidase staining kit (Pierce, Ill., USA) was used to carry out the ABC staining method. By so doing, sections were incubated with biotinylated horse anti-mouse Ig-G for 30 min, followed by two rinses in PBS, and then incubated with avidin-biotinylated peroxidase complex for 30 min more. Finally, sections were rinsed in PBS and incubated with 3-3′-diaminobenzidine (Sigma-Aldrich) and 0.01% hydrogen peroxide for 15 min. Slides were washed with PBS, dehydrated in ascending alcohol concentrations, cleared in xylene and coverslipped in DPX-EXLI mounting medium.

TH-inmunoreactive (TH-IR) cell bodies were counted (10×, brightfield) in three consecutive sections per animal. The counting started at the first section where SNc was clearly separated from the ventral tegmental area by the medial terminal nucleus of the accessory optic tract. The optical densities of the TH-IR fibers in the striatum were measured in three slices per animal of the rostral level of the striatum, corresponding to the area around the second 6-OHDA injection. Sections were placed under a microscope connected via a video camera to a computer. Quantitative image analysis were performed with MCID computerized image analysis system (St Catherines, Ontario, Canada). The measured values (optical densities) were averaged for each rat and then expressed as relative percent from intact striatum of control animals.

In situ hybridization histochemistry

The oligonucleotides used were complementary to the following base sequences (GeneBank accession number in brackets): rat preprodynorphin, bases 607–654 [NM_019374]; rat preprodynorphin, bases 489–533 [NM_019374]; rat preproenkephalin, bases 513–542 [K02807]; human adenosine A_{2A} receptor, bases 285–329 [NM_00675]. They were custom-synthesized by Amersham Pharmacia Biotech (UK). The oligonucleotides were labeled at their 3′-end by using [$\alpha-^{33}$P]dATP (Amersham, UK) and terminal deoxynucleotidyl-transferase (Roche Molecular Biochemicals, Mannheim, Germany). Labeled probes were purified trough QIAquick Nucleotide Removal columns (Qiagen, Germany).

For in situ hybridization, frozen tissue striatal sections were brought to room temperature, air-dried, and fixed for 20 min in 4% paraformaldehyde in phosphate-buffered saline (1× PBS: 2.6 mM KCl, 1.4 mM KH_2PO_4, 136 mM NaCl, and 8 mM Na_2HPO_4), washed once in 3×PBS, twice in 1×PBS, 5 min each, and incubated in a freshly prepared solution of predigrested pronase (Calbiochem, San Diego, Calif., USA) at a final concentration of 20 IU/ml in 50 mM TrisHCl pH 7.5, 5 mM EDTA for 2 min at 20°C. Proteolytic activity was stopped by inmersion for 30 s in 2 mg/ml glycine in PBS. Tissues were rinsed in PBS and dehydrated in graded ethanol 2 min each. For hybridization, labeled probes were diluted to a final concentration of 10^7 cpm/ml in a solution containing 50% formamide, 4×SSC (1×SCC: 150 mM NaCl, 15 mM sodium citrate), 1×Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 1% sarkosyl, 10% dextran sulphate, 20 mM phosphate buffer, pH 7.0, 250 μg/ml yeast tRNA, and 500 μg/ml salmon sperm DNA. Tissues were covered with 100 μl of the hybridization solution and overlaid with Nescofilm (Bando Chemical, Kobe, Japan) coverslips to prevent evaporation. Sections were incubated in humid boxes overnight at 42°C and then washed 4 times (45 min each) in 600 mM NaCl, 20 mM TrisHCl, pH 7.5, 1 mM EDTA at 60°C. Hybridized sections were exposed to BIOMAX-MR film (Kodak) for 15 days depending on the probe used at −70°C with intensifying screens.

The specificity of the nucleotide hybridization signals was assessed as follows. For a given oligonucleotide probe the presence of a 50-fold excess of the same unlabeled oligonucleotide in the hybridization buffer

resulted in the abolishment of the specific hybridization signal (data not shown). The thermal stability of the hybrids was examined by washing a series of consecutive hybridized sections at increasing temperatures. Specific hybridization signals were still present in sections washed at 70°C but they were completely absent from sections washed at 80°C. No such decrease was observed in the background levels of the signal (data not shown).

The striatum were divided into two portions for the mRNA expression measurement, including the dorsolateral and the ventromedial striatum (Carta et al. 2002). Quantitative image analysis were performed with MCID computerized image analysis system (St Catherines).

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons. The level of statistical significance was set at $P < 0.05$ for all analysis.

Results

Substantia nigra cell counts

We assessed the effect of the adenosine A_{2A} receptor antagonist CSC on the 6-OHDA-induced dopaminergic neuronal degeneration in rats. In this experiment, CSC or vehicle were subacutely (7 days) administered before striatal 6-OHDA lesion. Striatal 6-OHDA administration induced a decrease in the number of TH-IR neurons in the ipsilateral SNc of the vehicle-treated group by 48% in comparison with contralateral SNc ($P < 0.01$). Sham-lesioned animals did not show any difference between both sides. Interestingly, CSC subacute pretreatment conferred a significant attenuation of the

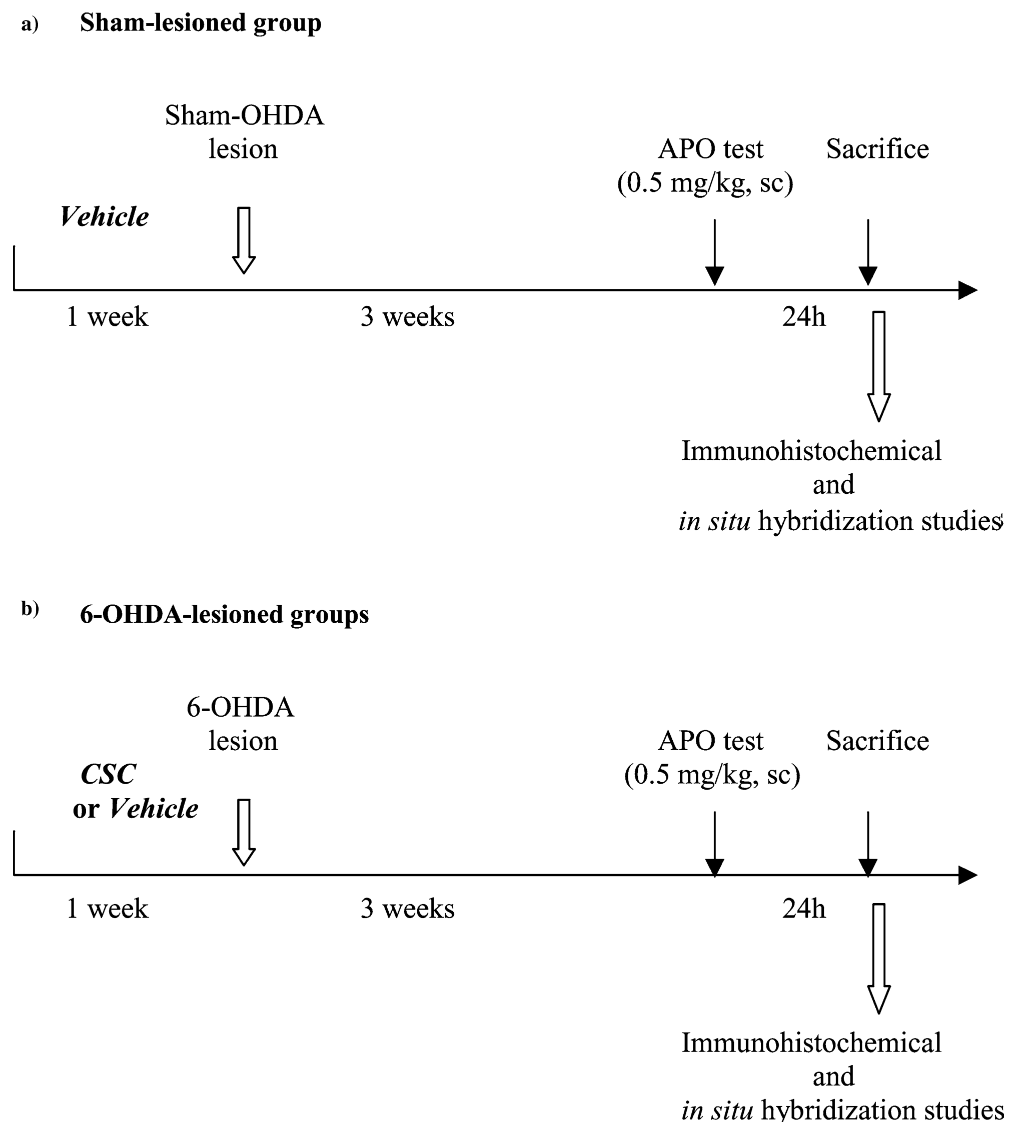

Fig. 1 Time-course of experiments described in the text

6-OHDA-induced decrease in the number of TH-IR neurons by about 12% ($P < 0.05$) (Fig. 1).

Striatal TH-immunoreactivity

In the vehicle-treated group, TH-IR decreased after striatal 6-OHDA administration by 20% in the ipsilateral striatum in comparison to contralateral striatum ($P < 0.01$), while in the sham-lesioned animals no changes in the TH-IR was observed. Subacute CSC pretreatments failed to attenuate the decrease in the TH-IR induced by striatal 6-OHDA administration ($P < 0.05$) (Fig. 2).

Rotational behavior

Apomorphine induced rotational behavior to the 6-OHDA-lesioned vehicle-treated animals ($P < 0.01$). Sham-lesioned animals did not show rotational behavior after apomorphine administration. Subacute CSC pretreatment did not modify the rotational behavior achieved by the 6-OHDA lesioned animals (Table 1).

Striatal adenosine A_{2A} receptor mRNA expression

Levels of the different mRNA were measured in the dorsolateral (DL) and ventromedial (VM) portion of the striatum (Fig. 3). Striatal 6-OHDA administration induced a significant increase of A2A receptor mRNA levels in the VM, but not in the DL lesioned striatum, compared with the sham-lesioned animals ($P < 0.05$). Subacute CSC pretreatment prevented this increase of A2A receptor mRNA levels induced by the striatal 6-OHDA administration ($P < 0.05$) (Figs. 4 and 5).

Striatal preproenkephalin mRNA expression

A significant increase in PPE mRNA levels in the DL lesioned striatum was caused by the 6-OHDA administration in the vehicle-treated animals compared with the sham-lesioned animals ($P < 0.01$). In the VM striatum, 6-OHDA administration did not induce any change on PPE mRNA levels. Subacute CSC treated animals did not modify the increase in PPE mRNA expression induced by 6-OHDA in the DL striatum (Fig. 6, 7).

Striatal preprodynorphin mRNA expression

Striatal 6-OHDA administration induced no changes in PPD mRNA levels neither in the DL or in the VM striatum in the vehicle-treated animals compared with the sham-lesioned animals. Subacute CSC pretreatment produced a significant decrease in mRNA PPD levels in the lesioned VM striatum compared with sham-lesioned animals ($P < 0.05$) (Fig. 8, 9, 10).

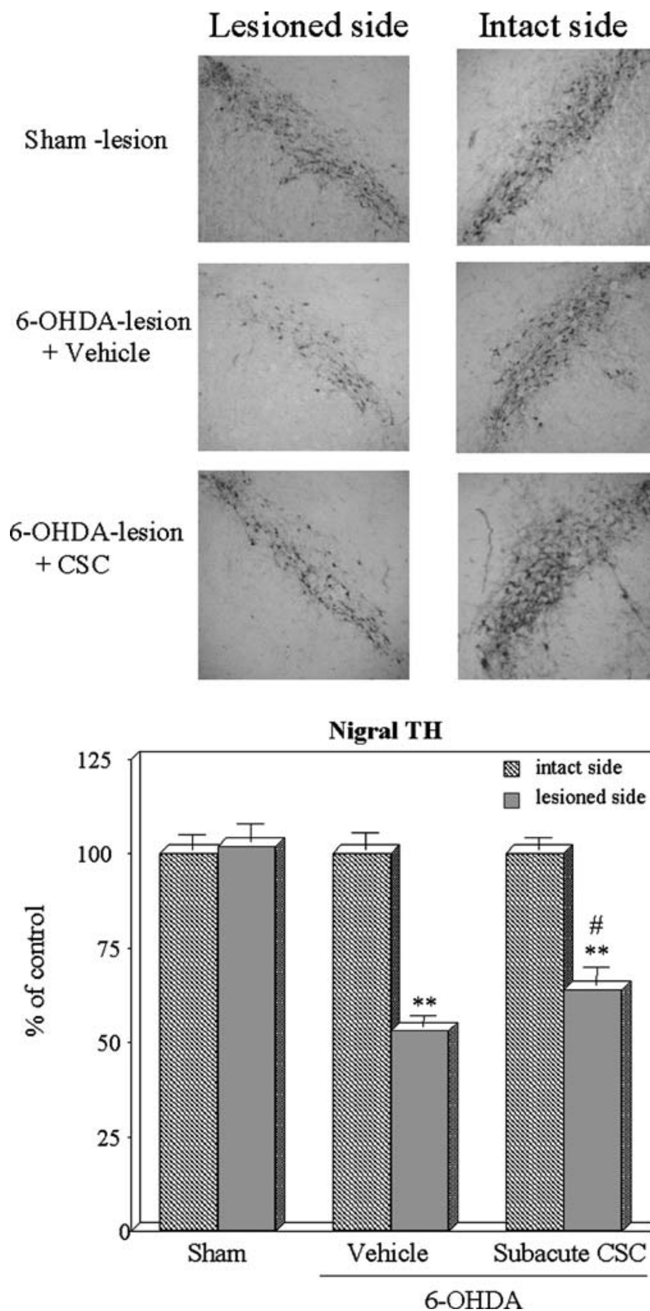

Fig. 2 Effect of unilateral striatal 6-OHDA-induced lesion and CSC pretreatment on nigral TH-IR. *Upper*: a representative TH immunohistochemistry. *Bottom*: subacute administration of the A_{2A} antagonist CSC partially attenuated the decrease in TH-IR when administered for 7 days until 30 min before 6-OHDA administration. Vehicle-treated group received a solution of 2% DMSO IP Sham-lesioned group received intrastriatal administration of 0.2% ascorbic acid/saline. Values are expressed as mean ± SEM.** $P < 0.01$ vs intact side. # $P < 0.05$ vs vehicle-treated animals

Discussion

In the present study, a four-site terminal lesion resulted in a partial loss of TH-positive fibers in the striatum, leading to a retrograde degeneration of the 48% of

Table 1 Effect of subacute administration of the adenosine A_{2A} antagonist CSC on apomorphine-induced rotations

Treatments	Apomorphine-induced rotations
Sham	-1.6 ± 23
Vehicle + 6-OHDA	187 ± 43*
Subacute CSC + 6-OHDA	212 ± 62

dopaminergic neurons in the SNc. Systemic CSC administration partially attenuated nigral dopaminergic cell loss induced by intrastriatal 6-OHDA administration. These results are in agreement with previous reports that demonstrated a neuroprotective effect of A_{2A} antagonists in excitoxicity (Jones et al. 1998a,b; Behan and Stone 2002) and in ischemia models (Von Lubitz et al. 1995; Bona et al. 1997; Monopoli et al. 1998; Melani et al. 2003). Moreover, it has been recently described a neuroprotective effect of A_{2A} receptor blockade in experimental models of PD since it has been shown that caffeine and selective A_{2A} antagonists such as CSC, but not A_1 antagonists, attenuated MPTP toxicity in mice (Chen et al. 2001, 2002; Xu et al. 2002). In 6-OHDA-treated rats, the selective A_{2A} antagonist KW-6002 has shown to protect against both the loss of nigral dopaminergic cells and the degeneration of its terminals (Ikeda et al. 2002). In the present study, CSC administration did not attenuate the decrease in striatal TH-IR induced by intrastriatal 6-OHDA indicating a lack of protection of striatal dopaminergic terminals.

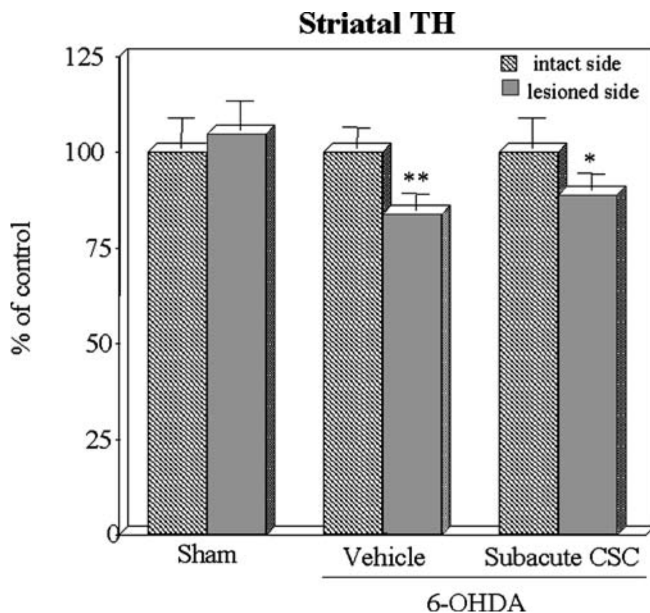

Fig. 3 Effect of unilateral striatal 6-OHDA-induced lesion and CSC pretreatment on striatal TH-IR. Subacute administration of the A_{2A} antagonist CSC did not attenuate the decrease in TH-IR when administered for 7 days until 30 min before 6-OHDA administration. Vehicle-treated group received a solution of 2% DMSO IP Sham-lesioned group received intrastriatal administration of 0.2% ascorbic acid/saline. Values are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$ vs intact side

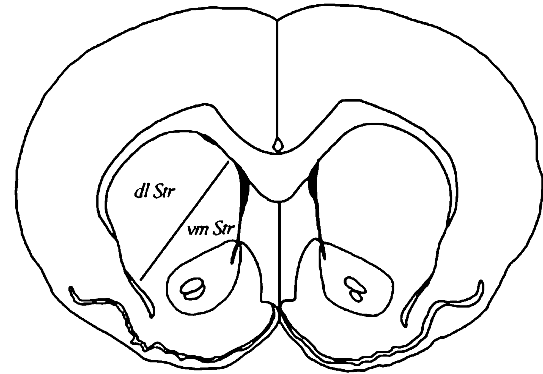

Fig. 4 Schematic representation of striatal portions considered to measure mRNA expression by in situ hybridization

This result agrees with the observation that the rotational behavioral showed by the group of animals pretreated with CSC did not differ from the vehicle-treated animals. Two methodological differences need to be taking in account to interpret the different results in comparison to a previous report (Ikeda et al. 2002). First

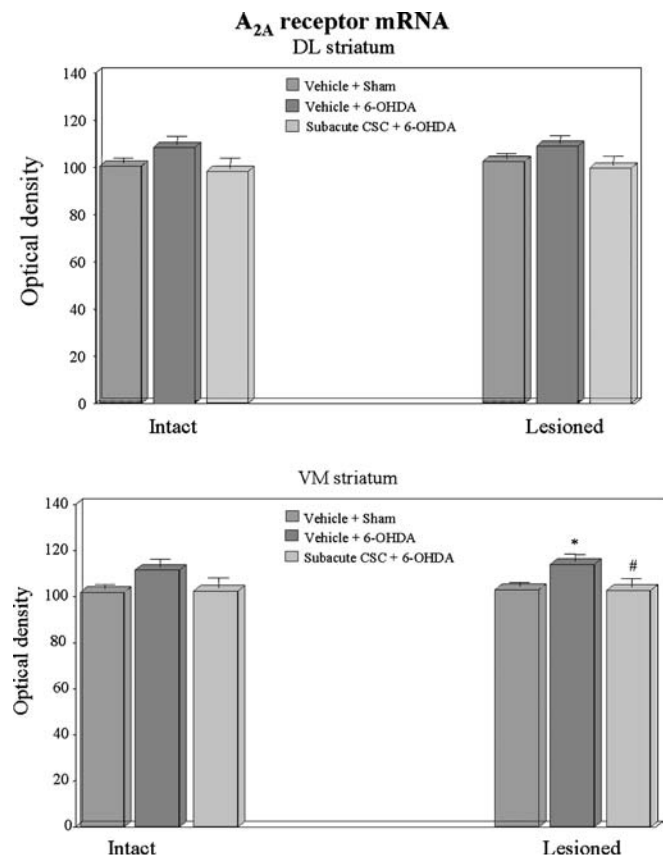

Fig. 5 Effect of unilateral striatal 6-OHDA-induced lesion and CSC pre-treatment on DL (*upper*) and VM (*bottom*) striatal adenosine A_{2A} mRNA expression. Subacute administration of the A_{2A} antagonist CSC attenuated the increase in A_{2A} mRNA expression in the VM striatum induced by intrastriatal 6-OHDA lesion. Values are expressed as mean ± SEM. *$P < 0.05$ vs sham-lesioned animals, #$P < 0.05$ vs vehicle-treated animals

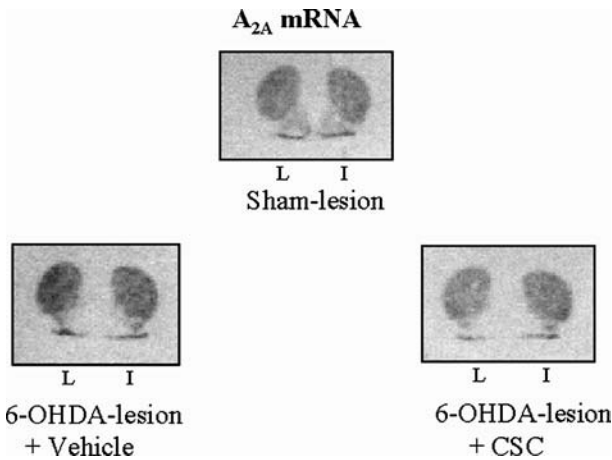

Fig. 6 Representative film autoradiograms of coronal brain sections (14 μm) showing striatal A_{2A} mRNA labeling in control (sham-lesioned), vehicle-treated and CSC-treated rats

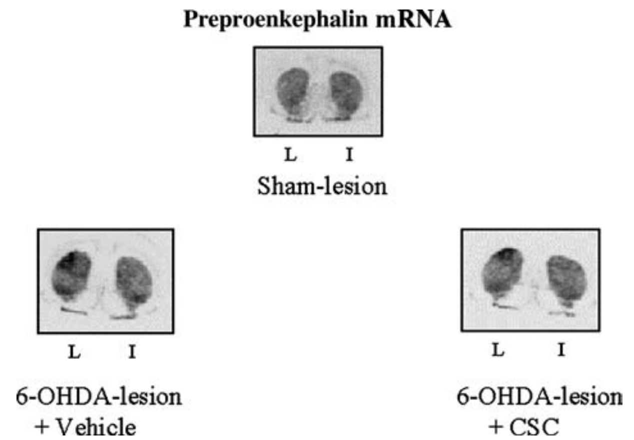

Fig. 8 Representative film autoradiograms of coronal brain sections (14 μm) showing striatal PPE mRNA labeling in control (sham-lesioned), vehicle-treated and CSC-treated rats

of all, a much higher total dose of 6-OHDA has been used in the present study and it has been injected at four different sites of the striatum and not at a single one. In fact, a four-site 6-OHDA lesion has been compared with a manifest symptomatic stage in PD, whereas one-site 6-OHDA injection causes more restricted presymptomatic

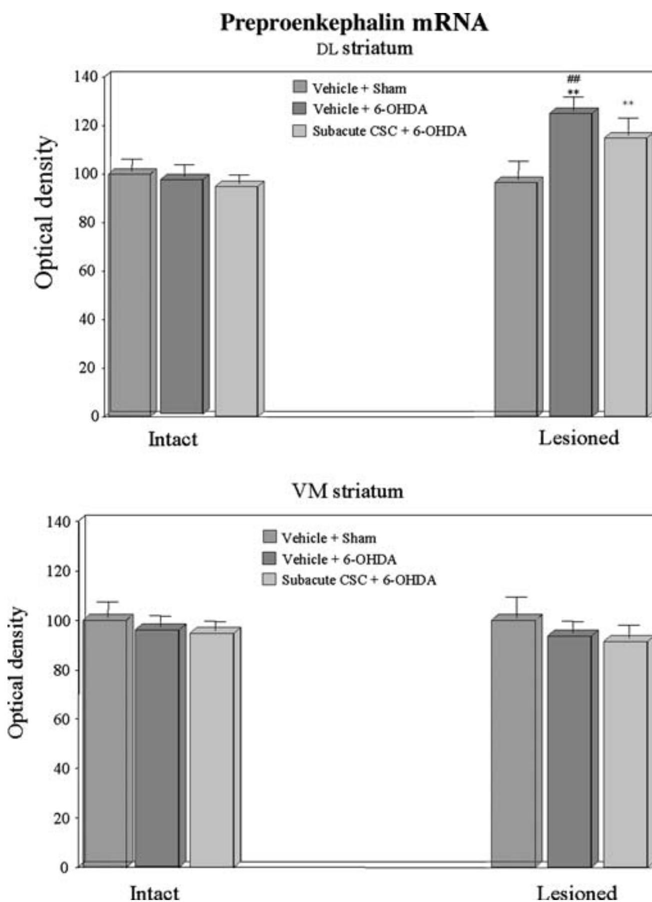

Fig. 7 Effect of unilateral striatal 6-OHDA-induced lesion and CSC pretreatment on DL (*upper*) and VM (*bottom*) striatal PPE mRNA expression. Subacute administration of the A_{2A} antagonist CSC did not attenuate the increase in PPE mRNA expression in the DL striatum induced by intrastriatal 6-OHDA lesion. Values are expressed as mean ± SEM. $**P < 0.01$ vs sham-lesioned animals

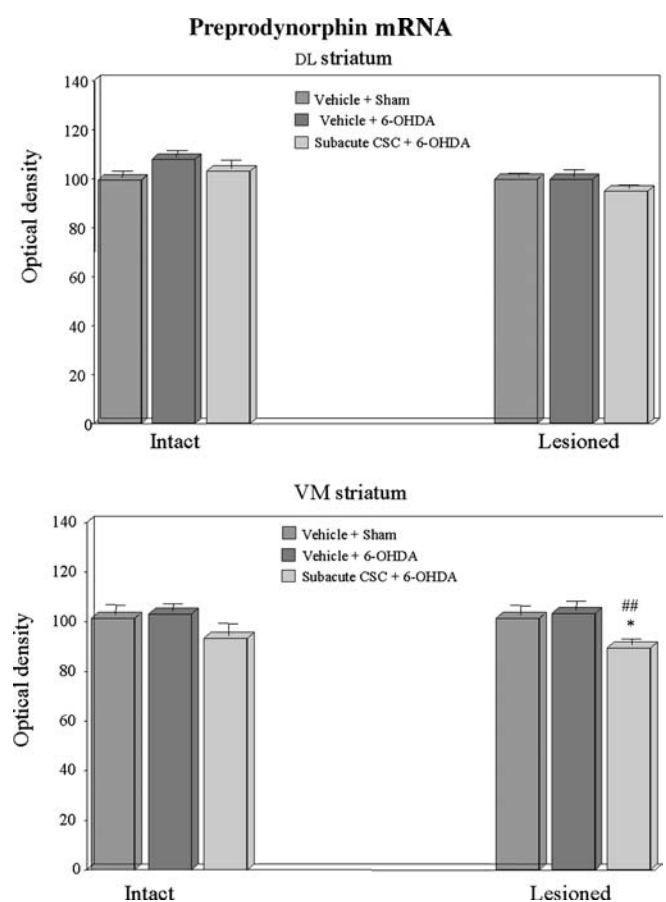

Fig. 9 Effect of unilateral striatal 6-OHDA-induced lesion and CSC pretreatment on DL (*upper*) and VM (*bottom*) striatal PPD mRNA expression. Subacute administration of the A_{2A} antagonist CSC decreased PPD mRNA expression in the VM striatum. Values are expressed as mean ± SEM. $*P < 0.05$ vs sham-lesioned animals; $\#P < 0.01$ versus vehicle-treated animals

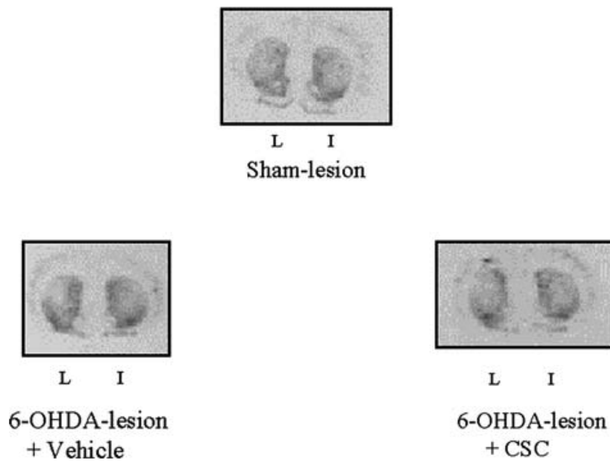

Fig. 10 Representative film autoradiograms of coronal brain sections (14 μm) showing striatal PPD mRNA labeling in control (sham-lesioned), vehicle-treated and CSC-treated rats

lesions (Kirik et al. 1998). The second methodological difference is the treatment protocol used since in the present study CSC was subacutely administered for 7 days until 30 min before 6-OHDA lesion. However, in the work of Ikeda et al. (2002), the A_{2A} antagonist, KW-6002 was administered before the 6-OHDA administration and during 1 week later.

The precise mechanisms underlying the neuroprotective effect of A_{2A} antagonists are still not known. Since there are evidences of the existence of functional A_{2A} receptor in nigral dopaminergic neurons, it is possible that these neurons might be the site of the neuroprotective action by A_{2A} antagonists (Okada et al. 1996; Chen et al. 2000). However, different A_{2A} receptor-mediated mechanisms may be involved in central actions of A_{2A} antagonists. For example, A_{2A} receptor stimulation enhances striatal glutamate extracellular levels (Simpson et al. 1992; Popoli et al. 1995; Sebastiao and Ribeiro 1996) and the A_{2A} antagonist SCH 58261 decreases both spontaneous and K^+-evoked striatal glutamate outflow in rats (Corsi et al. 2000). Since glutamate is considered to play a major role inducing ischemia and post-ischemia cell death (Choi and Rothman 1990), protective effects of A_{2A} -receptor antagonists against ischemic injury may be attributed to their ability to reduce excitatory amino acid outflow.

Several previous studies have involved A_{2A} receptors in cerebral inflammation (Sullivan et al. 1999) and therefore adenosine might contribute to the pathological changes in PD by triggering the activation of surrounding glial cells, which are known to appear around degenerating dopaminergic neurons in PD (Hirsch et al. 1999) since A_{2A} receptor-mediated mechanisms have been described in substantia nigra (Alfinito et al. 2003). Although A_{2A} receptors inhibit the production of several pro-inflammatory cytokines (Dianzani et al. 1994), they can also potentiate the pro-inflammatory effect of those compounds (Scholz-Pedretti et al. 2001). Activation of A_{2A} receptors can promote glial proliferation after brain injury (Hindley et al. 1994; Rathbone et al. 1999) and enhances nitric oxide and cyclooxygenase production in vitro (Fiebich et al. 1996). However, another report suggests that adenosine may inhibit astroglial activation (Michael et al. 1999). The protective effect of A_{2A} receptor antagonists may therefore reflect a net attenuation of pro-inflammatory activity.

CSC is also a potent and selective inhibitor of monoamine oxidase-B (MAO-B) (Chen et al. 2002) and it has been suggested that the neuroprotective effect of this drug may be due to a blockade of the conversion of MPTP to MPDP+, an oxidation mediated by MAO-B, in the MPTP model of PD (Chen et al. 2002). The generation of reactive oxygen species induced by 6-OHDA may arise from two distinct mechanisms, namely deamination by MAO oxidation or auto-oxidation (Blum et al. 2001). Thus, 6-OHDA, like DA, may be a substrate for MAO (Breese and Taylor 1971; Karoum et al. 1993). An involvement of MAO in 6-OHDA-induced neurotoxicity has been suggested following the observation that the MAO inhibitor, selegiline, prevents 6-OHDA toxicity (Salonen et al. 1996) and, consequently, the inhibition of MAO by CSC could be one explanation for the CSC neuroprotective effects.

The restricted expression of A_{2A} receptors in the striatum and the lack of evidence for their expression on dopaminergic neurons themselves (Rosin et al. 1998; Svenningsson et al. 1999) suggest that A_{2A} receptors modulation of dopaminergic neurotoxicity is indirect either by an alteration in their retrograde neurotrophic influence in nigrostriatal neurons (Siegel and Chauhan 2000) or more likely through a feedback circuit running back to the dopaminergic nigral neurons (Rodriguez et al. 1998). In the latter case, stimulation of A_{2A} receptors on striatopallidal neurons enhances GABA release in the globus pallidus (Mayfield et al. 1996) and thus may facilitate the indirect pathway disinhibition of STN activity, which in turn through the glutamatergic projections to the SNc may contribute to excitotoxic injury of dopaminergic neurons (Piallat et al. 1996). Inactivation of A_{2A} receptors, on the other hand, would prevent the proposed dopaminergic toxicity produced through this circuit.

In order to investigate the possible involvement of the indirect and the direct striatopallidal pathways activity changes in the neuroprotection induced by CSC administration we have study the expression of striatal mRNA expression for adenosine A_{2A} receptor, PPE and PPD in rats with a striatal 6-OHDA-induced lesion. We have shown that 6-OHDA intrastriatal administration produce a significant increase in adenosine A_{2A} receptor mRNA expression in the VM striatum, but not in the DL, ipsilateral to the lesion. These results are in agreement with a recent report (Pinna et al. 2002) in which the expression of adenosine A_{2A} receptor mRNA was increased in the striatum in association with a decrease in striatal extracellular levels of adenosine. The increase

was selectively detected in the lateral portion of the lesioned striatum which partially overlaps the portion that in the present study has been defined as VM striatum. As has been proposed (Pinna et al. 2002), the specific distribution of A_{2A} receptors to the lateral portion of the striatum may account for the lack of changes in A_{2A} mRNA expression when the whole striatum was studied (Kaeling-Lang et al. 2000). Binding studies have failed to demonstrate a modification of A_{2A} receptor after 6-OHDA-induced denervation (Alexander and Reddington 1989; Martinez-Mir et al. 1991; Morelli et al. 1994; Przedbordki et al. 1995). These discrepancies between receptor binding and hybridization have been attributed to different sensitivities of the two methodologies (Pinna et al. 2002). In the present study, CSC pretreatment prevented the A_{2A} receptor mRNA up-regulation in the VM striatum. This result suggests that the neuroprotective effect of CSC might be induced by an attenuation of the increased activity of the indirect pathway in which neuronal A_{2A} receptors are expressed.

With the objective to investigate whether the attenuation of the hyperactivity of the indirect pathway is involved in the neuroprotective effect of A_{2A} antagonism we have studied the expression of PPE mRNA, since its increase has been correlated to the hyperactivity of this pathway (Young et al. 1986; Gerfen et al. 1990; Cadet et al. 1992; Asselin et al. 1994). We have shown that striatal 6-OHDA administration increased the PPE mRNA levels in the DL lesioned striatum in agreement with previous descriptions after striatal (Winkler et al. 2002) and after nigrostriatal lesions induced by 6-OHDA (Young et al. 1986; Gerfen et al. 1990; Cadet et al. 1992; Zeng et al. 1995) or MPTP administration (Augood et al. 1989; Asselin et al. 1994; Jolkkonen et al. 1995). The most relevant finding in the present study is that CSC pretreatment did not attenuate this increase in PPE mRNA in the DL lesioned striatum. Since the increase in PPE mRNA may reflect an overactivity of the striatopallidal indirect pathway leasing to increased inhibition of pallidal neurons and subsequent overactivity of STN (Levy et al. 1997; Parent et al. 2000), the results obtained in the present work suggest that the neuroprotective effect of A_{2A} antagonist CSC is not related to an attenuation of the indirect striatopallidal pathway.

In the present study, no modification of PPD mRNA levels has been induced by intrastriatal 6-OHDA lesion in agreement with the level of denervation of the lesioned striatum as previously showed (Winkler et al. 2002). CSC pretreatment induced a decrease in the expression of dynorphin mRNA in the VM striatum in rats with a striatal 6-OHDA-induced lesion. The role of this decrease in the expression of PPD in the VM lesioned striatum is not known. The VM striatum appears to play a critical role in mediating motoric effects (Boye et al. 2001; Ikemoto 2002; Ikemoto and Witkin 2003). It has been suggested that the A_{2A} receptors, localized in the ventral striatum play a key role in the modulation of motor activity. Barraco et al. (1993) showed that the local infusion in the VM of the selective A_{2A} agonist CGS21680, but not a selective A_1, induced a pronounced motor depressant in mice. As far as the VM striatum is concerned, low doses of caffeine stimulate spontaneous motor activity (Svenningsson et al. 1995). Morphological observations suggest that GABAergic striopallidal neurons and strionigral-strioentopeduncular neurons might be the main locus for A_{2A}-D_2 and A_1-D_1 interactions, respectively (Schiffmann et al. 1991; Fink et al. 1992). The two subtypes of GABAergic efferent neurons are also present in the VM striatum (LeMoine and Bloch 1995), although with a less well-defined separation of their target brain areas. Although A_{2A} and D_1 receptor are not located on the same striatal efferent neurons, there are several studies that clearly illustrate an A_{2A} receptor modulation of the striatonigral pathway at behavioral and biochemical level in 6-OHDA-lesioned rats (Morelli et al. 1994; Pinna et al. 1996; Pollack and Fink 1996). It has been shown that systemic administration of the A_{2A} antagonist SCH 58261 caused a decrease in the number of c-fos mRNA-containing neurons in the striatum not only in the striatopallidal pathway but in the striatonigral pathway (Le Moine et al. 1997). A_{2A} receptor antagonism-induced potentiation of D_1 receptor-mediated motor activation has been demonstrated (Pinna et al. 1996). All these effects could be explained by an interaction at the network level, similar to the synergistic effect of dopamine D_1 and D_2 agonists (Robertson and Robertson 1986; Paul et al. 1992).

Since synaptic connections between spiny neurons of the direct and indirect pathways have been described (Aronin et al. 1986; Yung et al. 1996; Seeman and Tallerico 2003), A_{2A} antagonists could modulate the direct pathway via the indirect pathway. The existence of such functional interaction between adenosine A_{2A} receptors and dopamine D_1 receptors may underlie the effect of the administration of CSC diminishing PPD mRNA expression in the VM striatum shown in the present study. Furthermore, the increase of dynorphin mRNA levels seen after chronic levodopa treatment in 6-OHDA lesioned mice is not seen in A_{2A} knockout mice (Freduzzi et al. 2002), demonstrating that A_{2A} receptors are involved in dynorphin mRNA levels modulation and therefore in striatonigral pathway activity. These results are in agreement with our results showing that A_{2A} blockade attenuates dynorphin expression. The role of this decrease in PPD mRNA expression in the VM striatum on the neuroprotective effect of CSC is not known. However, a cytotoxic effect of dynorphin has been described (McIntosh et al. 1994; Hauser et al. 1999; Tan-No et al. 2001). Thus, it might be speculated that a decrease in dynorphin might have a neuroprotective effect.

In summary, the present results show that the neuroprotective effect of the adenosine A_{2A} antagonist CSC in striatal 6-OHDA-lesioned rats does not result from a normalization of the increase in striatal PPE mRNA expression in the DL striatum suggesting that other

different mechanisms may be involved. A recent hypothesis of a different role of A_{2A} receptors at pre- versus postsynaptic sites on neuroprotection needs to be taken in account, since it has been shown (Tebano et al. 2004) that whereas effects of presynaptic A_{2A} receptors are potentially detrimental, the effects of postsynaptic A_{2A} receptors are potentially beneficial.

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