

Effect of Adenosine A_{2A} Receptor Antagonists and L-DOPA on Hydroxyl Radical, Glutamate and Dopamine in the Striatum of 6-OHDA-Treated Rats

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Abstract A_{2A} adenosine receptor antagonists have been proposed as a new therapy of PD. Since oxidative stress plays an important role in the pathogenesis of PD, we studied the effect of the selective A_{2A} adenosine receptor antagonists 8-(3-chlorostyryl)caffeine (CSC) and 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385) on hydroxyl radical generation, and glutamate (GLU) and dopamine (DA) extracellular level using a microdialysis in the striatum of 6-OHDA-treated rats. CSC (1 mg/kg) and ZM 241385 (3 mg/kg) given repeatedly for 14 days decreased the production of hydroxyl radical and extracellular GLU level, both enhanced by prior 6-OHDA treatment in dialysates from the rat striatum. CSC and ZM 241385 did not affect DA and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA) extracellular levels in the striatum of 6-OHDA-treated rats. L-DOPA (6 mg/kg) given twice daily for two weeks in the presence of benserazide (3 mg/kg) decreased striatal hydroxyl radical and glutamate extracellular level in 6-OHDA-treated rats. At the same time, L-DOPA slightly but significantly increased the extracellular levels of DOPAC and HVA. A combined repeated administration of L-DOPA and CSC or ZM 241385 did not change the effect of L-DOPA on hydroxyl radical production and glutamate extracellular level in spite of an enhancement of extracellular DA level by CSC and elevation of extracellular level of DOPAC and HVA by ZM 241385. The data suggest that the 6-OHDA-induced damage of nigrostriatal DA-terminals is related to

oxidative stress and excessive release of glutamate. Administration of L-DOPA in combination with CSC or ZM 241385, by restoring striatal DA-glutamate balance, suppressed 6-OHDA-induced overproduction of hydroxyl radical.

Keywords Parkinson's disease · Oxidative stress · Adenosine A_{2A} receptor antagonists

Introduction

Parkinson's disease (PD) is a consequence of specific progressive neurodegeneration of substantia nigra (SN) pars compacta dopaminergic neurons accompanied by depletion of dopamine (DA) in the striatum and SN (Fahn and Sulzer 2004). The underlying motivation for the search for new antiparkinsonian drugs stems from limitations of PD therapy with L-DOPA, e.g., dyskinesias, declining efficacy in the course of the treatment (Cenci 2007), as well as L-DOPA toxicity (Chen et al. 2008). Recently, a new therapy with adenosine A_{2A} receptor antagonists has been proposed for the treatment of PD (Schwarzschild et al. 2006). Some behavioral studies revealed that A_{2A} antagonists alone and in combination with L-DOPA mitigated motor deficits in animal models of PD (Schwarzschild et al. 2006; Bishnoi et al. 2007; Morelli et al. 2007). The promising data obtained in non-human primates (Kanda et al. 1998) helped researchers to launch clinical trials with A_{2A} antagonists in PD patients (Xu et al. 2005). The mechanism by which A_{2A} antagonists alleviate parkinsonian motor dysfunctions is based on modulation of DA responses through the existing A_{2A}-D₂ receptor interactions (Schwarzschild et al. 2006) and their ability to modulate GABA release and DA-dependent *c-fos*

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activation in the indirect striatopallidal pathway (Pollack and Fink 1995; Ochi et al. 2000). By counteracting D₂ receptor function, presynaptic A_{2A} receptors are able to control corticostriatal glutamatergic transmission (Tozzi et al. 2007).

Epidemiological studies have indicated an inverse relationship between the consumption of caffeine, a non-selective adenosine receptor antagonist, and the risk of developing PD (Ross et al. 2000; Ascherio et al. 2001). A protective effect of caffeine and more selective antagonists of A_{2A} receptors, similar to genetic inactivation of A_{2A} receptors, was observed in an animal MPTP neurotoxicity model (Xu et al. 2005; Chen et al. 2007) or in ischemia and excitotoxic brain injury models (Popoli et al. 2004; Chen et al. 2007). The mechanism allowing A_{2A} antagonists to protect dopaminergic neurons has not been fully explained yet, but a variety of their effects on various types of neurons, e.g., glutamatergic nerve terminals and glial or immune cells, suggest its complex nature (Chen et al. 2007). Since oxidative stress is regarded as the main factor contributing to the etiology of PD, it seems of crucial importance to find out whether A_{2A} adenosine antagonists may influence the production of free radicals in nigrostriatal neurons.

The present study was aimed at investigating the efficacy of A_{2A} antagonists in counteraction of oxidative stress resulting from the disturbed DA-glutamate balance in the animal model of PD based on 6-hydroxydopamine (6-OHDA) administration. The effectiveness of a synergistic combination of L-DOPA and an A_{2A} antagonist, shown in animal models (Wardas et al. 2001) and in parkinsonian patients (Xu et al. 2005) to counteract symptoms of PD, points to the usefulness of A_{2A} antagonists as a supplement to L-DOPA therapy. Therefore, the effect of the combination of an A_{2A} antagonist and L-DOPA on cellular production of hydroxyl radicals was also determined with the use of microdialysis in freely moving animals.

Materials and Methods

Animals

Microdialysis studies were conducted on male Wistar rats (250–300 g), bred at the Institute of Pharmacology, Polish Academy of Sciences, Krakow, Poland. The rats were housed in temperature- and humidity-controlled rooms on a 12-h light/dark cycle, with free access to filtered tap water and standard pelleted laboratory chow throughout the study. The experimental procedures and housing conditions used were in strict accordance with the Polish legal regulations concerning experiments on animals (Dz. U. 05.33.289). All the experimental protocols were approved

by the Local Bioethics Commission for Animal Experiments.

Drugs

L-3,4-dihydroxyphenylalanine (L-DOPA), 6-hydroxydopamine (6-OHDA), 8-(3-chlorostyryl)caffeine (CSC), benserazide, haloperidol, and *p*-hydroxybenzoic acid (PBA) were obtained from Sigma-Aldrich (Poznań, Poland), whereas 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385) came from TOCRIS (Warsaw, Poland). All the chemicals used for HPLC were purchased from Merck (Warsaw, Poland). L-DOPA and benserazide were dissolved in saline. A solution of PBA was prepared in an artificial cerebrospinal fluid (aCSF) and was then adjusted to pH = 7.4 with 0.1 M NaOH. CSC was initially dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Poznań, Poland) and was then diluted in at least 20 vols. of the vehicle consisting of a 20:80 v/v mixture of Alkamulus EL-620 (Rhone-Poulenc, Cranbury, NJ) and a phosphate-buffered saline. ZM 241385 was dissolved in a small amount of DMSO and was then diluted in Cremophor EL (Sigma-Aldrich, Poznań, Poland) and 0.9% NaCl (final concentration: a 15% DMSO and a 15% Cremophor EL). All injections were made through an intraperitoneal route (i.p.) 14 days after 6-OHDA administration. CSC (1 mg/kg) and ZM 241385 (3 mg/kg) were given once a day for 13 days. L-DOPA was given twice a day in a dose of 6 mg/kg, together with benserazide (3 mg/kg), for 13 days to eliminate diurnal fluctuations in brain DA level. The last doses of the A_{2A} receptor antagonists CSC and ZM 241385 were given 20 min before the injection of a challenging dose of L-DOPA (12 mg/kg) with benserazide (6 mg/kg). Control animals received respective vehicles.

6-OHDA Lesions

Animals were anesthetized with a combination of ketamine (75 mg/kg) and xylazine (10 mg/kg) and given an unilateral stereotaxic injection of 12 µg/µl 6-OHDA dissolved in 0.9% NaCl containing 0.05% ascorbic acid into the left medial forebrain bundle (MFB) by means of BAS (USA) infusion pump. Stereotaxic injections were placed 1.9 mm anterior to the interaural line, 1.8 mm lateral to the midline, and 8.2 mm ventral to the dura, according to the atlas of Paxinos and Watson (1998). Two weeks after the 6-OHDA injection, the behavior was assessed by monitoring body rotations induced by apomorphine (0.05 mg/kg s.c.). The number of contralateral rotations was recorded for 60 min and, only animals which made 200 turns in 1 h were included for further experimental steps.

Monoamine Levels' Determination

For the measurement of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA), the brain tissue was homogenized in 0.1 M HClO₄, centrifuged at 4°C for 5 min at 10,000 g, and the supernatant was filtered through 0.1 μm (Millipore) membranes. 2–5 μl of sample was then injected into high-performance liquid chromatograph (HPLC) with electrochemical detection.

In Vivo Microdialysis

The rats were anaesthetized with ketamine (75 mg/kg i.m.) and xylazine (10 mg/kg i.m.) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). Their skulls were exposed, and small holes were drilled for the insertion of microdialysis probes into the striatum using the following coordinates: 1.8 mm anterior from the bregma; 2.8 mm lateral from the sagittal suture; and -7.0 mm ventral from the dura (Paxinos and Watson 1998). Vertical microdialysis probes were constructed as described in detail elsewhere (Gołębiewska et al. 2009). Probe inlets were connected to a syringe pump (BAS, IN, USA) which delivered an aCSF composed of [mM]: NaCl 147, KCl 4.0, MgCl₂ 1.0, CaCl₂ 2.2; pH = 7.4 at a flow rate of 1.5 μl/min. All metal parts of the aCSF delivery system were replaced with PEEK components or were passivated with 6 M HNO₃. Baseline samples were collected every 20 min after the washout period to obtain a stable extracellular neurotransmitter level. Appropriate drugs were then administered 20 min before L-DOPA injection given at time 0, as shown in figures, and dialysate fractions were collected for 240 min. At the end of the experiment, the rats were killed and their brains were histologically examined to validate probe placement.

Analytic Procedure

DA, DOPAC, and HVA were analyzed using HPLC with an electrochemical detection. The level of hydroxyl radicals was estimated as 3,4-dihydroxybenzoic acid (3,4-DHBA), a product of the spin trap reagent PBA (1 mM) applied via microdialysis probe. DA and its metabolites were simultaneously determined in the same fractions of striatal dialysates. Chromatography was performed using an LC-10 AD pump (Shimadzu Europa GmbH, Warsaw, Poland), an LC-4B amperometric detector with a cross-flow detector cell (BAS, IN, USA), and a BDS-Hypersil C18 analytical column (3 × 100 mm, 3 μm; Thermo Electron Corp., UK). The mobile phase consisted of 0.1 M monochloroacetic acid adjusted to pH = 3.7 with

3 M sodium hydroxide, 0.5 mM EDTA, 13 mg/l 1-octanesulfonic acid sodium salt, a 5.7% methanol, and a 0.8% acetonitrile. The flow rate was 0.5 ml/min, and the applied potential of a 3-mm glassy carbon electrode was +600 mV at a sensitivity of 2 nA/V. Concentrations of all compounds were calculated by comparing their peak areas with respective standards and were processed by Chromax 2001 (Pol-Lab, Warsaw, Poland) software run on a personal computer. The obtained values were not corrected for in vitro probe recovery, which was approximately 10–15%.

Glutamate was measured in dialysates (20 μl) after derivatization with 4-dimethylaminoazobenzene-4'-sulfonylchloride (DABS-Cl) at 70°C for 12 min, according to Knecht and Chang (1986). Dabsylated amino acids were separated on an Ultrasphere ODS (4.6 × 150 mm, 3 μm) column (Supelco, Poznań, Poland) by gradient elution, with solvent A (10 mM citric acid, 4% dimethylformamide) and solvent B (acetonitrile). Dabsylated compounds were detected by measuring an absorbance at 436 nm using Beckman Amino Acid System Gold with VIS detection.

Data Analysis

All obtained data are given in absolute numbers. The statistical significance of differences between experimental groups was calculated using a one-way ANOVA for repeated-measures, followed by Tukey's *post-hoc* test. The results were considered statistically significant at $P < 0.05$.

Results

Effects of 6-OHDA on DA, DOPAC, HVA, and Glutamate in the Rat Striatum

Unilateral injection of 6-OHDA (12 μg/μl) into the left medial forebrain bundle produced a substantial damage of nigrostriatal neurons 2 weeks after administration

Table 1 Tissue content of DA, DOPAC and HVA in the striatum and substantia nigra of control and 6-OHDA (12 μg/side)-treated rats

Striatum pg/mg w.t. mean ± SEM (n)			
Treatment	DA	DOPAC	HVA
Control	11856 ± 823 (8)	1322 ± 78 (8)	774 ± 74 (8)
6-OHDA	93 ± 115 (8)**	62 ± 35 (8)**	77 ± 35 (8)**
Substantia nigra pg/mg w.t. mean ± SEM (n)			
Control	1371 ± 80 (10)	342 ± 32 (10)	128 ± 22 (10)
6-OHDA	543 ± 78 (15)**	88 ± 3,8 (15)**	42 ± 3,1 (15)**

** $P < 0.01$ versus control

Table 2 Extracellular concentration of DA, DOPAC, HVA and glutamate in control and 6-OHDA (12 µg/side)-treated rats

Treatment	Mean ± SEM (n)			pmoles/10 µl
	pg/10 µl			
Control	DA	DOPAC	HVA	Glutamate
Control	8.83 ± 0.62 (8)	2478 ± 260 (8)	1788 ± 150 (8)	2.94 ± 0.15 (8)
6-OHDA	0.73 ± 0.06(8)**	31 ± 6 (8)**	29 ± 4 (8)**	6.19 ± 0.55(8)**

** $P < 0.01$ versus control

(Tables 1, 2). The contents of DA, DOPAC, and HVA were markedly decreased by ca. 99, 95, and 90 percent, respectively in ipsilateral striatum (Table 1). Significant decreases in DA, DOPAC, and HVA contents (by 60, 74, and 67 percent, respectively) were also observed in the left substantia nigra (Table 1). 6-OHDA injection attenuated extracellular levels of DA, DOPAC, and HVA (by ca. 91, 99 and 98 percent, respectively) and increased the extracellular level of striatal glutamate (Table 2).

Effects of CSC and ZM 241385 on Hydroxyl Radical Productions and Extracellular Levels of Glutamate, DA, DOPAC, and HVA in 6-OHDA Treated Rats

The last doses of CSC (1 mg/kg) and ZM 241385 (3 mg/kg) in animals with nigrostriatal neurons damaged by 6-OHDA and treated chronically with A_{2A} antagonists for 13 days significantly decreased the production of hydroxyl radical (Fig. 1a). Repeated measures ANOVA showed a significant effect of treatment ($F_{3,18} = 9.22$, $P = 0.0006$), a significant effect of time ($F_{14,252} = 37.24$, $P = 0$), and interaction was found between the two factors ($F_{42,252} = 7.05$, $P = 0$). Post-hoc analysis with Tukey's test showed that hydroxyl radical level in 6-OHDA-treated animals was significantly increased when compared with intact rats ($P < 0.01$). Hydroxyl radical level's decrease caused by CSC and ZM 241385 was significant in comparison with 6-OHDA group ($P < 0.05$).

The last doses of CSC (1 mg/kg) and ZM 241385 (3 mg/kg) in animals with nigrostriatal neurons damaged by 6-OHDA and treated chronically with A_{2A} antagonists for 13 days did not influence extracellular effects of DA, DOPAC, and HVA (Fig. 2a, b, c). In contrast, both antagonists significantly decreased extracellular level of glutamate, which was markedly increased in 6-OHDA-treated animals (Fig. 1b). Repeated measures ANOVA showed a significant effect of treatment ($F_{3,20} = 41.43$, $P = 0$), but a non-significant effect of time ($F_{14,280} = 1.01$, $P = 0.44$), and no interaction between both factors was found ($F_{42,280} = 0.42$, $P = 0.99$). Post-hoc analysis with Tukey's test showed that glutamate level was significantly decreased by CSC and ZM 241385 ($P < 0.01$) in comparison with 6-OHDA group, while

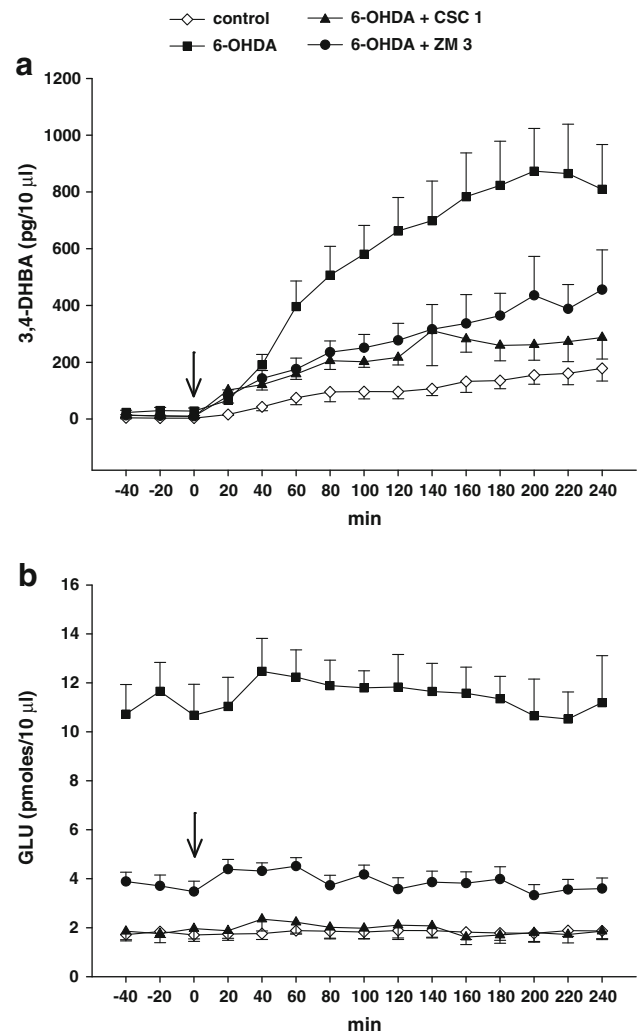


Fig. 1 Effects of CSC (1 mg/kg \times 14) and ZM 241385 (ZM, 3 mg/kg \times 14) on extracellular concentration of 3,4-DHBA (a) and GLU (b) in the striatum of rats treated with 6-OHDA. The injection of the last dose of the drug is indicated by an arrow. The data are the mean \pm SEM ($n = 6-8$). **a** $P < 0.01$ 6-OHDA, 6-OHDA + ZM 3 versus control; $P < 0.05$ 6-OHDA versus 6-OHDA + CSC 1, 6-OHDA + ZM 3. **b** $P < 0.01$ 6-OHDA versus control; $P < 0.01$ 6-OHDA versus 6-OHDA + CSC 1, 6-OHDA + ZM 3

there was a significant increase in glutamate level in animals with nigrostriatal neurons damaged by 6-OHDA ($P < 0.01$ versus naïve animals) (Fig. 1b).

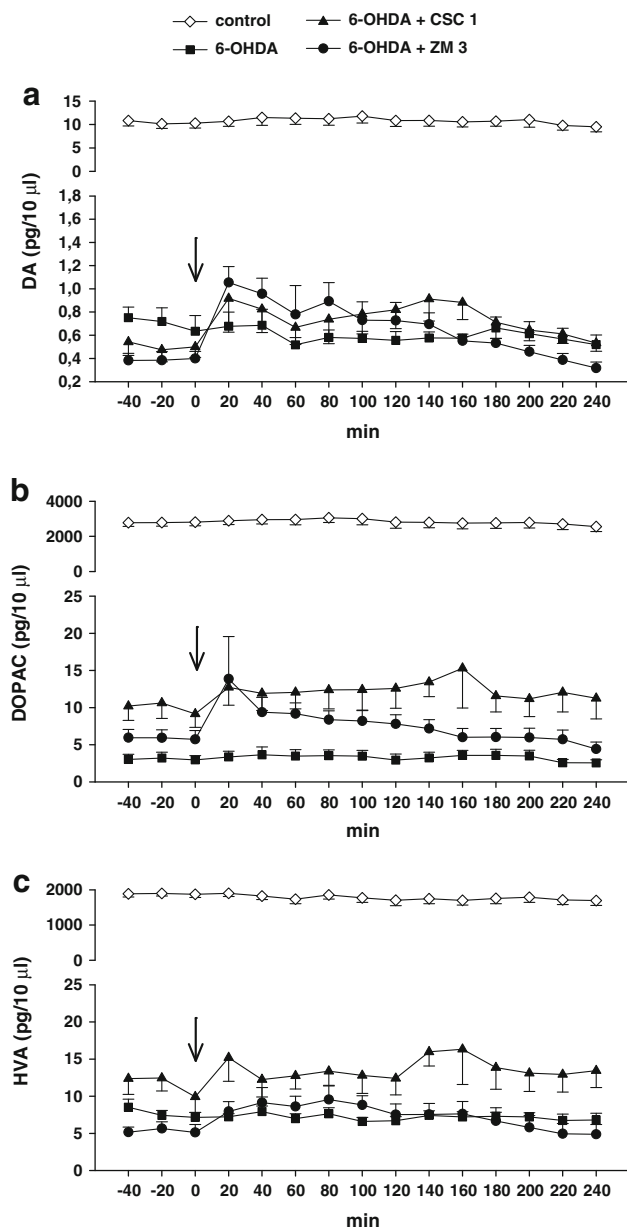


Fig. 2 Effects of CSC (1 mg/kg \times 14) and ZM 241385 (ZM, 3 mg/kg \times 14) on extracellular concentrations of DA (a), DOPAC (b), and HVA (c) in the striatum of rats treated with 6-OHDA. The injection of the last dose of the drug is indicated by an arrow. The data are the mean \pm SEM ($n = 6-8$). a, b, c $P < 0.01$ 6-OHDA, 6-OHDA + CSC 1, 6-OHDA + ZM 3 versus control

Effects of CSC and ZM 241385 Administered Together with L-DOPA on Hydroxyl Radical Productions and Extracellular Levels of Glutamate, DA, DOPAC, and HVA in 6-OHDA-Treated Rats

The last dose of L-DOPA (12 mg/kg) in rats lesioned with 6-OHDA and treated chronically with L-DOPA (6 mg/kg twice daily for 13 days) decreased hydroxyl radical production to control level. The last doses of CSC and ZM

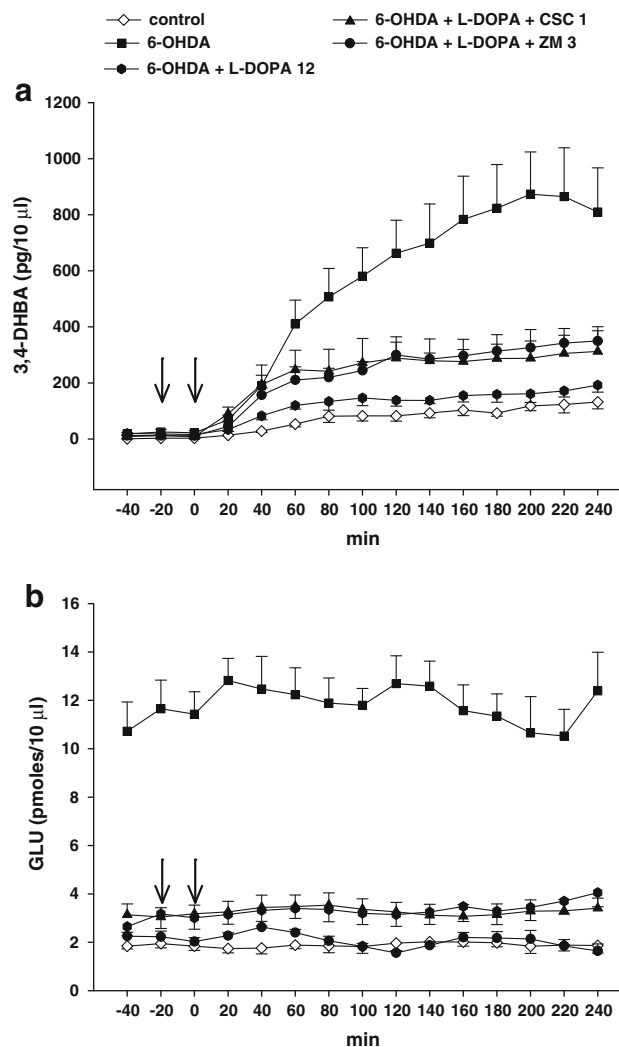


Fig. 3 Effects of CSC (1 mg/kg \times 14) and ZM 241385 (ZM, 3 mg/kg \times 14) given in combination with L-DOPA (6 mg/kg \times 14, twice daily) on extracellular concentrations of 3,4-DHBA (a) and GLU (b) in the striatum of rats treated with 6-OHDA. The time of injection of the last doses of CSC, and ZM 241385 given 20 min before challenging dose of L-DOPA (12 mg/kg) is indicated by an arrow. The data are the mean \pm SEM ($n = 6-8$). a $P < 0.01$ 6-OHDA, 6-OHDA + L-DOPA, 6-OHDA + L-DOPA + CSC 1, 6-OHDA + L-DOPA + ZM 3 versus control. b $P < 0.01$ 6-OHDA, 6-OHDA + L-DOPA, 6-OHDA + L-DOPA + CSC 1, 6-OHDA + L-DOPA + ZM 3 versus control

241385 did not influence the L-DOPA-decreased hydroxyl radical level (Fig. 3a). There was a significant effect of treatment ($F_{4,24} = 9.90$, $P = 0.0001$), a significant effect of time ($F_{14,336} = 47.55$, $P = 0$), and interaction was found between both factors ($F_{56,336} = 8.85$, $P = 0$). Post-hoc analysis with Tukey's test showed that the effect of L-DOPA on hydroxyl radical level was significant in comparison with 6-OHDA group ($P < 0.001$) (Fig. 3a).

Similarly, a decrease in extracellular level of glutamate was observed under the influence of L-DOPA. The last

doses of CSC and ZM 241385 did not change the effect of L-DOPA on glutamate extracellular level (Fig. 3b). There was a significant effect of treatment ($F_{4,23} = 49.43$, $P = 0$), but not effect of time ($F_{14,322} = 0.61$, $P = 0.86$), and there was no interaction between both factors ($F_{56,322} = 0.71$, $P = 0.94$). Post-hoc analysis with Tukey's test showed that the effects of L-DOPA and a combination of L-DOPA with CSC 1 and ZM 241385 3 were significant in comparison with 6-OHDA group ($P < 0.01$) (Fig. 3b).

The last dose of L-DOPA (12 mg/kg) in rats lesioned with 6-OHDA and treated chronically with L-DOPA (6 mg/kg twice daily for 13 days) markedly increased extracellular levels of DA, DOPAC, and HVA. The last dose of CSC (1 mg/kg) in combination with L-DOPA elevated extracellular levels of DA, but did not affect extracellular level of DOPAC and HVA (Fig. 4a, b, c). The last dose of ZM 241385 (3 mg/kg) in combination with L-DOPA increased extracellular level of DOPAC and HVA, while did not change extracellular level of DA (Fig. 4a, b, c). There was a significant effect of treatment [DA: $F_{4,25} = 26.09$, $P = 0$; DOPAC: $F_{4,26} = 88.32$, $P = 0$; HVA: $F_{4,25} = 177.9$, $P = 0$], time [DA: $F_{14,350} = 19.47$, $P = 0$; DOPAC: $F_{14,364} = 12.02$, $P = 0$; HVA: $F_{14,350} = 8.56$, $P = 0$], and interaction was found between both factors [DA: $F_{56,350} = 4.10$, $P = 0$; DOPAC: $F_{56,364} = 1,84$, $P = 0.0005$; HVA: $F_{56,350} = 8.56$, $P = 0$]. Post-hoc analysis with Tukey's test showed that effect of L-DOPA was significant in comparison with 6-OHDA group (DA, DOPAC: $P < 0.05$, HVA: $P < 0.01$) (Fig. 4a, b, c). The effect of CSC on DA level was significant in comparison with L-DOPA-treated animals ($P < 0.05$). ZM 241385 significantly increased extracellular effect of DOPAC and HVA in comparison with L-DOPA group ($P < 0.05$) (Fig. 4a, b, c).

Discussion

The findings of the present study indicate that adenosine A_{2A} receptor antagonists, CSC, and ZM 241385 given repeatedly in doses that effectively reduced haloperidol-induced catalepsy (Gołombiowska et al. 2009) inhibited generation of hydroxyl radical in the striatum of rats with damaged nigrostriatal neurons. Furthermore, it was shown that L-DOPA itself also lowered the production of hydroxyl radical and this effect was not changed by CSC and ZM 241385 administered repeatedly with L-DOPA.

In our study, unilateral administration of 6-OHDA into the MFB nearly completely damaged nigrostriatal neurons, as we observed a decrease in striatal extracellular DA level by ca. 92% and a decrease in extracellular levels of

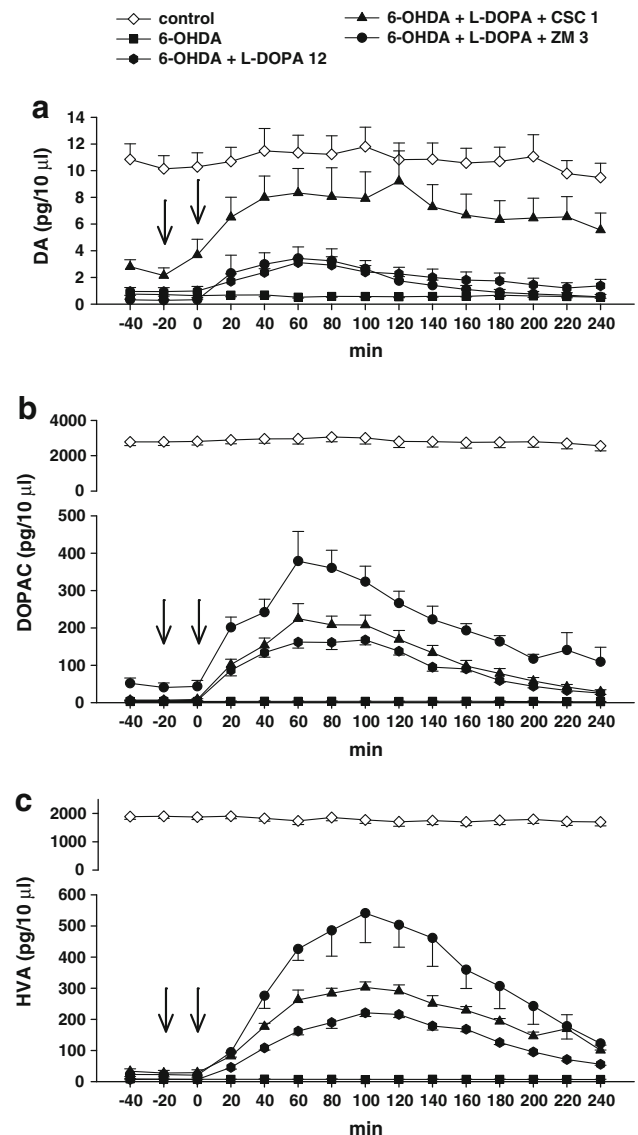


Fig. 4 Effects of CSC (1 mg/kg \times 14) and ZM 241385 (ZM, 3 mg/kg \times 14) given in combination with L-DOPA (6 mg/kg \times 14, twice daily) on extracellular concentration of DA (**a**), DOPAC (**b**), and HVA (**c**) of rats treated with 6-OHDA. The time of injection of the last doses of CSC and ZM 241385 given 20 min before challenging dose of L-DOPA (12 mg/kg) is indicated by an arrow. The data are the mean \pm SEM ($n = 6-8$). **a** $P < 0.01$ 6-OHDA, 6-OHDA + L-DOPA, 6-OHDA + L-DOPA + ZM 3 versus control; $P < 0.05$ 6-OHDA + L-DOPA + CSC 1 versus control; $P < 0.05$ 6-OHDA + L-DOPA versus 6-OHDA + L-DOPA + CSC 1. **b** $P < 0.01$ 6-OHDA, 6-OHDA + L-DOPA, 6-OHDA + L-DOPA + CSC 1, 6-OHDA + L-DOPA + ZM 3 vs. control; $P < 0.01$ 6-OHDA + L-DOPA, 6-OHDA + L-DOPA + CSC 1, 6-OHDA + L-DOPA + ZM 3 versus 6-OHDA; $P < 0.01$ 6-OHDA + L-DOPA + ZM 3 versus 6-OHDA + L-DOPA. **c** $P < 0.01$ 6-OHDA, 6-OHDA + L-DOPA, 6-OHDA + L-DOPA + CSC 1, 6-OHDA + L-DOPA + ZM 3 versus control; $P < 0.01$ 6-OHDA + L-DOPA, 6-OHDA + L-DOPA + CSC 1, 6-OHDA + L-DOPA + ZM 3 versus 6-OHDA; $P < 0.01$ 6-OHDA + L-DOPA + ZM 3 versus 6-OHDA + L-DOPA

DOPAC and HVA by 99% in comparison with sham-operated animals. Tissue contents of DA, DOPAC, and HVA also decreased in the striatum (below 90–99%) and in the substantia nigra (below 60–74%). It indicated that 6-OHDA given into the MFB, damaged not only striatal DA terminals, but also DA cell bodies in the substantia nigra. In addition, we found a marked increase in striatal extracellular glutamate level, which indicates that DA exerts inhibitory control of glutamate release and deficit in DA synthesis disturbs glutamate-DA balance. These data are in line with the results of other authors, who also observed an increase in glutamate release in brain of rats injected intracerebrally with 6-OHDA (Meshul et al. 1999, 2000; Jonkers et al. 2002).

It is known that DA is a very good source of free radicals, which are formed during enzymatic metabolism of DA by MAO, or by its non-enzymatic autoxidation that leads to production of very reactive DA quinones and semi-quinones (Halliwell 2006). However, under conditions of a marked DA deficit, i.e., conditions resembling late-stage PD, glutamate might be the source of free radicals. It was found that an excessive release of glutamate from nerve terminals causes overstimulation of glutamate receptors, calcium overload in cytosol, and stimulation of intracellular signaling in the process called excitotoxicity (Dunnet and Björklund 1999). An increased calcium influx into cytosol disturbs mitochondrial functions and in particular the mitochondrial respiratory chain. This leads to a leakage of superoxide radical from mitochondrial complexes and, after cellular defense system failure, to oxidative stress (Dunnet and Björklund 1999). Our study shows that a marked increase in striatal extracellular glutamate level under conditions of DA deficit in rats treated with 6-OHDA might be the reason for an enhanced formation of hydroxyl radical. Multiple doses of CSC and ZM 241385 lowered extracellular glutamate level to values seen in control animals, and at the same time, they decreased the production of hydroxyl radical. Thus, we may suggest that the effect of A_{2A} antagonists on glutamate level and subsequent inhibition of free radical generation underlies the neuroprotective mechanism of these drugs. Observations of other authors, who showed neuroprotective effects of A_{2A} antagonists in a global and focal cerebral ischemia model (Phillis 1995; Von Lubitz et al. 1995; Chen et al. 1999; Melani et al. 2003) and glutamate or quinolic acid-induced excitotoxicity (Popoli et al. 2002; Pintor et al. 2004) are in line with our data. Similarly, other studies reported that ZM 241385, SCH 58261, and CSC counteracted the kainate and quinolic acid-induced neuronal damage (Jones et al. 1998; Behan and Stone 2002).

In this study, L-DOPA given repeatedly at low doses significantly inhibited both the formation of hydroxyl

radical and striatal extracellular glutamate level. L-DOPA also slightly but significantly increased the extracellular levels of DA, DOPAC, and HVA in the striatum of rats with damaged nigrostriatal neurons. These data show that low doses of L-DOPA administered in the late-stage PD may restore the disturbed DA-glutamate balance, even when the concentration of DA does not achieve control values. In addition, under conditions of a long-term DA deficit, supersensitive DA receptors stimulated by low concentration of L-DOPA-derived DA may inhibit overactive glutamate neurons. The latter observation we have made is very interesting in the light of controversies related to L-DOPA toxicity. The argument is that in some in vitro and in vivo experimental models, e.g., following rotenone (Nakao et al. 1997) or 6-OHDA administration (Clerehugh et al. 1999, Ishida et al. 2000), it was shown that oxidative stress may be the cause of L-DOPA toxicity. On the other hand, some reports have indicated neuroprotective effects, since L-DOPA given systematically acutely or repeatedly, as well as by intrastriatal infusion, diminished free radical production in rats with a unilateral 6-OHDA lesion of nigrostriatal neurons (Camp et al. 2000). The lack of L-DOPA toxic effects after its long-term administration was also observed by Fornai et al. (2000) in MPTP-treated mice. Similar results were reported by Dziejczapolski et al. (1997) and Kostrzewa et al. (2000) in rats lesioned with 6-OHDA. In contrast to our earlier study, in which we showed the formation of free radicals after chronic L-DOPA administration in the striatum of normal rats, the results obtained in the present study on rats with damaged DA neurons indicate neuroprotective properties of L-DOPA. In our opinion, L-DOPA's neuroprotective versus toxic effect depends on the stage of damage to DA neurons, amount of DA synthesized and the rate of its metabolism. CSC and ZM 241385 administered into rats in combination with L-DOPA only slightly and not significantly, enhanced the production of hydroxyl radical. At the same time, the extracellular level of L-DOPA-derived DA was increased by CSC, while ZM 241385 accelerated metabolism of L-DOPA-derived DA. However, neither DA nor DOPAC or HVA levels achieved control values in the presence of CSC or ZM 241385. Furthermore, both drugs were ineffective in changing the extracellular glutamate level which was decreased by L-DOPA. Thus, subtle changes in DA synthesis or metabolism may influence the production of free radicals, but glutamate overflow seems to be critical for oxidative stress development in the late-stage PD. Recently, Aguiar et al. (2008) showed that combination of CSC and L-DOPA-decreased the glutamate release in 6-OHDA-lesioned rats. Furthermore, they reported that A_{2A} adenosine receptor blockade with CSC significantly decreased nitrate levels and lipid peroxidation in 6-OHDA-lesioned rats, pointing to antioxidant effect of the drug.

In summary, the obtained results indicate that neuroprotective mechanism of A_{2A} antagonists in the late-stage PD may be related to the inhibitory effect of these drugs on oxidative stress resulting from glutamate-induced excitotoxicity.

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References

- Aguiar LMV, Macêdo DS, Vasconcelos SMM, Oliveira AA, de Sousa FCF, Viana GSB (2008) CSC, an adenosine A_{2A} receptor antagonist and MAO B inhibitor, monoamine neurotransmission, and amino acid alterations in the 6-OHDA-lesioned rats. *Brain Res* 1191:192–199
- Ascherio A, Zhang SH, Hernán MA, Kawachi I, Colditz GA, Speizer FE, Willett WC (2001) Prospective study of caffeine consumption and risk of Parkinson's disease in men and women. *Ann Neurol* 50:56–63
- Behan WMH, Stone TW (2002) Enhanced neuronal damage by co-administration of quinolinic acid and free radicals, and protection by adenosine A_{2A} receptor antagonists. *Br J Pharmacol* 135:1435–1442
- Bishnoi M, Chopra K, Kulkarni SK (2007) Theophylline, adenosine receptor antagonist prevents behavioral, biochemical and neurochemical changes associated with an animal model of tardive dyskinesia. *Pharmacol Rep* 59:181–191
- Camp DM, Loeffler DA, LeWitt PA (2000) L-DOPA does not enhance hydroxyl radical formation in the nigrostriatal dopamine system of rats with a unilateral 6-hydroxydopamine lesion. *J Neurochem* 74:1229–1240
- Cenci MA (2007) Dopamine dysregulation of movement control in L-DOPA-induced dyskinesia. *Trends Neurosci* 30:236–243
- Chen J-F, Huang Z, Ma J, Zhu J, Moratalla R, Standaert D, Moskowitz MA, Fink JS, Schwarzschild MA (1999) A_{2A} adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. *J Neurosci* 19:9192–9200
- Chen J-F, Sonsalla PK, Pedata F, Melani A, Domenici MR, Popoli P, Geiger J, Lopes LV, de Mendonça A (2007) Adenosine A_{2A} receptors and brain injury: broad spectrum of neuroprotection, multifaceted actions and “fine tuning” modulation. *Prog Neurobiol* 83:310–331
- Chen L, Ding Y, Cagniard B, Van Laar AD, Mortimer A, Chi W, Hastings TG, Kang UJ, Zhuang X (2008) Unregulated cytosolic dopamine causes neurodegeneration associated with oxidative stress in mice. *J Neurosci* 28:425–433
- Cleren C, Vilpoux C, Dourmap N, Bonnet J-J, Costentin J (1999) Acute interactions between L-DOPA and neurotoxic effects of 1-methyl-4-phenylpyridinium or 6-hydroxydopamine in mice. *Brain Res* 830:314–319
- Dunnet SB, Björklund A (1999) Prospects for new restorative and neuroprotective treatments in Parkinson's disease. *Nature* 399(Supp):A32–A39
- Dziewczapolski G, Murer G, Agid Y, Gershanik OS, Raisman-Vozari R (1997) Absence of neurotoxicity of chronic L-DOPA in 6-hydroxydopamine-lesioned rats. *Neuroreport* 8:975–979
- Fahn S, Sulzer D (2004) Neurodegeneration and neuroprotection in Parkinson's disease. *NeuroRx* 1:139–154
- Fornai F, Battaglia G, Gessi M, Giorgi FS, Orzi F, Nicoletti F, Ruggieri S (2000) Time-course and dose-response study on the effects of chronic L-DOPA administration on striatal dopamine levels and dopamine transporter following MPTP toxicity. *Brain Res* 887:110–117
- Golembiowska K, Dziubina A, Kowalska M, Kamińska K (2009) Effect of adenosine A_{2A} receptor antagonists on L-DOPA-induced hydroxyl radical formation in rat striatum. *Neurotox Res* 15:155–166
- Halliwel B (2006) Oxidative stress and neurodegeneration: where are we now? *J Neurochem* 97:1634–1658
- Ishida Y, Hashiguchi H, Todaka K, Ishizuka Y, Mitsuyama Y (2000) Repeated administration of high dose levodopa enhances hydroxyl radical production in the rat striatum denervated with 6-hydroxydopamine. *Neurosci Lett* 290:33–36
- Jones PA, Smith RA, Stone TW (1998) Protection against kainate-induced excitotoxicity by adenosine A_{2A} receptor agonists and antagonists. *Neuroscience* 85:229–237
- Jonkers N, Sarre S, Ebinger G, Michotte Y (2002) MK801 suppresses the L-DOPA-induced increase of glutamate in striatum of hemiparkinsonian rats. *Brain Res* 926:149–155
- Kanda T, Jackson MJ, Smith LA, Pearce RK, Nakamura J, Kase H, Kuwana Y, Jenner P (1998) Adenosine A_{2A} antagonist: a novel antiparkinsonian agent that does not provoke dyskinesia in parkinsonian monkeys. *Ann Neurol* 43:507–513
- Knecht R, Chang JY (1986) Liquid chromatographic determination of amino acids after gas-phase hydrolysis and derivatization with (dimethylamino)azobenzenesulfonyl chloride. *Anal Chem* 58:2375–2379
- Kostrzewa RM, Kostrzewa JP, Brus R (2000) Dopaminergic denervation enhances susceptibility to hydroxyl radicals in rat neostriatum. *Amino Acids* 19:183–199
- Melani A, Pantoni L, Bordoni F, Gianfriddo M, Bianchi L, Vannucchi MG, Bertorelli R, Monopoli A, Pedata F (2003) The selective A_{2A} receptor antagonist SCH 58261 reduces striatal transmitter outflow, turning behavior and ischemic brain damage induced by permanent focal ischemia in the rat. *Brain Res* 959:243–250
- Meshul CK, Emre N, Nakamura CM, Allen C, Donhue MK, Buckman JF (1999) Time-dependent changes in striatal glutamate synapses following 6-hydroxydopamine lesion. *Neuroscience* 88:1–16
- Meshul CK, Cogen JP, Cheng H-W, Moore C, Krentz L, McNeill TH (2000) Alterations in rat striatal glutamate synapses following a lesion of the cortico- and/or nigrostriatal pathway. *Exp Neurol* 165:191–206
- Morelli M, Di Paolo T, Wardas J, Calon F, Xiao D, Schwarzschild MA (2007) Role of adenosine A_{2A} receptors in parkinsonian motor impairment and L-DOPA-induced motor complications. *Prog Neurobiol* 83:293–309
- Nakao N, Nakai K, Itakura T (1997) Metabolic inhibition enhances selective toxicity of L-DOPA toward mesencephalic dopamine neurons in vitro. *Brain Res* 777:202–209
- Ochi M, Koga K, Kurokawa M, Kase H, Nakamura J, Kuwana Y (2000) Systemic administration of adenosine A_{2A} receptor antagonist reverses increased GABA release in the globus pallidus of unilateral 6-hydroxydopamine-lesioned rats: a microdialysis study. *Neuroscience* 100:53–62
- Paxinos G, Watson C (1998) *The Rat Brain in Stereotaxic Coordinates*, Academic Press, San Diego
- Phillips JW (1995) The effects of selective A_1 and A_{2A} adenosine receptor antagonists on cerebral ischemic injury in the gerbil. *Brain Res* 705:79–84
- Pintor A, Galluzzo M, Grieco R, Pezzola A, Reggio R, Popoli P (2004) Adenosine A_{2A} receptor antagonists prevent the increase

- in striatal glutamate levels induced by glutamate uptake inhibitors. *J Neurochem* 89:152–156
- Pollack AE, Fink JS (1995) Adenosine antagonists potentiate D2 dopamine-dependent activation of Fos in the striatopallidal pathway. *Neuroscience* 68:721–728
- Popoli P, Pintor A, Domenici MR, Frank C, Tebano MT, Pèzzola L, Scarchilli L, Quarta D, Reggio R, Malchiodi-Albedi F, Falchi M, Massotti M (2002) Blockade of striatal adenosine A_{2A} receptor reduces, through a presynaptic mechanism, quinolic acid-induced excitotoxicity: possible relevance to neuroprotective interventions in neurodegenerative diseases of the striatum. *J Neurosci* 22:1967–1975
- Popoli P, Blum D, Pintor A, Tebano MT, Frank C, Gianfriddo M, Domenici MR, Schiffmann SN, Pedata F (2004) The controversial role of adenosine receptor antagonists as neuroprotective agents. *Curr Med Chem* 4:35–45
- Ross GW, Abbott RD, Petrovitch H, Morens DM, Grandinetti A, Tung K-H, Tanner CM, Masaki KH, Blanchette PL, Curb JD, Popper JS, White LR (2000) Association of coffee and caffeine intake with the risk of Parkinson disease. *JAMA* 283:2674–2679
- Schwarzschild MA, Agnati L, Fuxe K, Chen J-F, Morelli M (2006) Targeting adenosine A_{2A} receptors in Parkinson's disease. *Trends Neurosci* 29:647–654
- Tozzi A, Tschertter A, Belcastro V, Tantucci M, Costa C, Picconi B, Centonze D, Calabresi P, Borsini F (2007) Interaction of A_{2A} adenosine and D2 dopamine receptors modulates corticostriatal glutamatergic transmission. *Neuropharmacology* 53:783–789
- Von Lubitz DK, Lin RC, Jacobson KA (1995) Cerebral ischemia in gerbils: effects of acute and chronic treatment with adenosine A_{2A} receptor agonist and antagonist. *Eur J Pharmacol* 287:295–302
- Wardas J, Konieczny J, Lorenc-Koci E (2001) SCH 58261, an A_{2A} adenosine receptor antagonist, counteracts parkinsonian-like muscle rigidity in rats. *Synapse* 41:160–171
- Xu K, Bastia E, Schwarzschild M (2005) Therapeutic potential of adenosine A_{2A} receptor antagonists in Parkinson's disease. *Pharmacol Ther* 105:267–310