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Characterization of the antiparkinsonian effects of the new adenosine A_{2A} receptor antagonist ST1535: Acute and subchronic studies in rats

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Abstract

Antagonism of adenosine A_{2A} receptor function has been proposed as an effective therapy in the treatment of Parkinson's disease. Thus, the study of new adenosine receptor antagonists is of great importance for the potential use of these drugs in clinical practice. The present study evaluated effects of the new preferential adenosine A_{2A} receptor antagonist 2-butyl-9-methyl-8-(2*H*-1,2,3-triazol-2-yl)-9*H*-purin-6-ylamine (ST1535) in unilaterally 6-hydroxydopamine lesioned rats. Acute ST1535 dose-dependently potentiated contralateral turning behaviour induced by a threshold dose of L-3,4-dihydroxyphenylalanine (L-DOPA) (3 mg/kg i.p.), a classical test for antiparkinson drug screening. Subchronic (18 days, twice a day) ST1535 (20 mg/kg i.p.)+L-DOPA (3 mg/kg i.p.) did not induce sensitization to turning behaviour or abnormal involuntary movements during the course of treatment, indicating a low dyskinetic potential of the drug. Moreover, while subchronic administration of a fully effective dose of L-DOPA (6 mg/kg i.p.) significantly increased GABA synthesizing enzyme glutamic acid decardoxylase (GAD67), dynorphin and enkephalin mRNA levels in the lesioned striatum, subchronic ST1535 (20 mg/kg i.p.)+L-DOPA (3 mg/kg i.p.) did not modify any of these markers, although it induced a similar number of contralateral rotations at the beginning of treatment. Finally, acute administration of ST1535 (20 mg/kg i.p.) proved capable of reducing jaw tremors in tacrine model of Parkinson's disease tremor. Results showed that ST1535, in association with a low dose of L-DOPA, displayed antiparkinsonian activity similar to that produced by a full dose of L-DOPA without exacerbating abnormal motor side effects. Moreover, in agreement to other well characterized adenosine A_{2A} receptor antagonists, ST1535 features antitremorigenic effects.

Keywords: Parkinson; Dyskinesia; Tremor; Dopamine; Neurodegenerative disease

1. Introduction

The 'gold standard' pharmacological treatment for Parkinson's disease is represented by the administration of L-3,4-dihydroxyphenylalanine (L-DOPA). However, the search for new drugs that either alone or in association with L-DOPA are capable of counteracting the motor deficits that characterize the disease is becoming increasingly important due to the high incidence of motor side effects associated to long-term treatment with L-DOPA. Adenosine A_{2A} receptor antagonists have been proposed as an effective therapy in Parkinson's disease since preclinical and initial clinical studies have shown that these drugs increase the therapeutic efficacy of L-DOPA without exacerbating L-DOPA-associated side effects (Bara-Jimenez et al., 2003; Grondin et al., 1999; Hauser et al., 2003; Kanda et al., 2000; Morelli, 2003; Schwarzschild et al., 2006).

In 6-hydroxydopamine-lesioned rats, subchronic intermittent treatment with L-DOPA is associated with sensitization to contralateral turning behaviour and abnormal involuntary movements as well as to changes on neuronal activity similar to those associated to L-DOPA-induced dyskinesia in humans (Carta et al., 2002; Henry et al., 1998, 2003; Lundblad et al.,

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2002; Nielsen and Soghomonian, 2004; Pinna et al., 2006; Soghomonian et al., 1996). In contrast, treatments characterized by a lower dyskinetic potential, such as dopamine D_2 receptor agonists or adenosine A_{2A} receptor antagonists in association with a low dose of L-DOPA, while producing a similar behavioural response at the onset of subchronic treatment, induce no or low sensitization in contralateral turning, whereas abnormal involuntary movements are not sensitized (Carta et al., 2006; Delfino et al., 2004; Henry et al., 1998; Pinna et al., 2001). Thus, analysis of these two parameters might represent a valid model to evaluate drug-induced dyskinesia in hemiparkinsonian rats.

Subchronic L-DOPA-induced sensitized motor responses in unilaterally 6-hydroxydopamine lesioned rats are associated to an overexpression of markers such as GABA synthesizing enzyme glutamic acid decardoxylase (GAD67), dynorphin and enkephalin mRNAs in efferent neurons of the 6-hydroxydopamine lesioned striatum. On the contrary, no or low intensity changes on striatal mRNA expression are associated to motor effects of drugs with weak dyskinetic effects (Carta et al., 2002; Henry et al., 1999; Ravenscroft et al., 2004). Thus, correlation between striatal gene expression changes and motor behavioural response sensitization seems indicative of the dyskinetic potential of drugs showing antiparkinsonian properties.

Interestingly, recent results have indicated that adenosine A_{2A} receptor antagonists may be active in the rat model of drug-induced parkinsonian tremor as well as in tremor of Parkinson's disease patients (Chase et al., 2003; Correa et al., 2004; Simola et al., 2004).

At this stage of research on adenosine A_{2A} receptor antagonists it is fundamental that the number of efficacious adenosine A_{2A} receptor antagonists available for selection for clinical trials in Parkinson's disease patients be increased.

The aim of the present study was to evaluate the antiparkinsonian efficacy of the adenosine A_{2A} receptor antagonist, 2-butyl-9-methyl-8-(2*H*-1,2,3-triazol-2-yl)-9*H*-purin-6-ylamine (ST1535), a newly synthesized compound having high affinity for adenosine A_{2A} receptors (Minetti et al., 2005; Stasi et al., 2006). Acute effects of ST1535 and its dyskinetic potential after subchronic administration were evaluated by studying potentiation of turning behaviour induced by L-DOPA and sensitization of turning behaviour and abnormal involuntary movements. After subchronic administration, modifications on striatal mRNA expression for GAD67, dynorphin and enkephalin following ST1535+L-DOPA were compared to changes induced by L-DOPA alone. Moreover, the effects of acute administration of ST1535 on intensity of jaw tremors induced in normal rats by acute tacrine were evaluated as index of antitremorigenic activity.

2. Materials and methods

2.1. Subjects

Male Sprague Dawley rats (Charles River, Calco, Milan, Italy) were used in all experiments. Rats were maintained on a 12-h light–dark cycle (light on 08.00–20.00 h) with food and water available ad libitum. All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC).

2.2. 6-hydroxydopamine lesion

In order to lesion the dopaminergic nigrostriatal pathway, rats (275–300 g) were anesthetized with chloral hydrate (400 mg/kg i.p.), placed on a David Kopf Instrument (Tujunga, CA, USA) stereotaxic apparatus and injected unilaterally into the left medial forebrain bundle at coordinates $A=-2.2$, $L=+1.5$ from bregma, $V=-7.8$ from dura, according to the atlas of Pellegrino et al. (1979) with 6-hydroxydopamine–HCl (8 μg/4 μl of saline containing 0.05% ascorbic acid). Rats were pretreated with desipramine (10 mg/kg i.p.) to prevent damage to noradrenergic neurons.

2.3. Evaluation of turning behaviour and abnormal involuntary movements

Turning behaviour was measured by placing rats in plexiglas hemispherical bowls (50 cm diameter) with sawdust on the bottom and connecting them to an automated rotameter system capable of detecting the number of full (360°) rotations in any direction (Carnegie Medicine, Sweden). Rats were placed in each apparatus 30 min before drug administration in order to acclimatize and to extinguish any spontaneous turning behaviour.

Abnormal involuntary movements were measured visually during turning behavioural tests. According to their topographic distribution abnormal involuntary movements were classified into three subtypes: (1) axial abnormal involuntary movements: torsion of head, neck and trunk towards the side contralateral to the lesion in a still position; (2) limb abnormal involuntary movements: movements of the forelimb and the paw contralateral to the lesion (flexions and extensions of the forelimb and opening and closing of the digits); (3) orolingual abnormal involuntary movements: tongue protrusion and jaw movements, that were not observed during the course of the treatment. Each abnormal involuntary movement was quantified in 1 min testing-period and expressed as time spent by the rat in each movement.

2.4. Drug treatments

2.4.1. Acute L-DOPA and ST1535+L-DOPA treatment

Two weeks after the unilateral 6-hydroxydopamine lesion, rats were screened on the basis of their contralateral rotation in response to L-DOPA (50 mg/kg i.p.)+benserazide (30 mg/kg i.p.). Rats not showing at least 200 contralateral rotations during 3 h testing period were eliminated from the study. Three days later, rats were administered with L-DOPA (3 mg/kg i.p.)+benserazide (6 mg/kg i.p.) in combination with vehicle (10% dimethylsulfoxide (DMSO)+45% polyethylene glycol (PEG 400)+45% distilled water, i.p.) or with ST1535 (10, 20 or 40 mg/kg i.p.). L-DOPA was administered 5 min after vehicle or ST1535 injection, whereas benserazide was administered 30 min before L-DOPA injection. Contralateral rotations were measured every 10 min for 2 h.

2.4.2. Subchronic L-DOPA and ST1535+L-DOPA treatment

According to Pinna et al. (2001), three weeks after the unilateral 6-hydroxydopamine lesion, rats were screened on the basis of their contralateral rotation in response to L-DOPA (50 mg/

kg i.p.)+benserazide (30 mg/kg i.p.). Rats not showing at least 200 contralateral rotations during 3 h testing period were eliminated from the study. Seven days later, rats were divided into 4 groups and administered, twice a day (12-h interval), for 18 days, according to the following schedule: (I) vehicle (i.p.), (II) ST1535 (20 mg/kg i.p.), (III) vehicle (i.p.)+L-DOPA (6 mg/kg i.p.), (IV) ST1535 (20 mg/kg i.p.)+L-DOPA (3 mg/kg i.p.). L-DOPA was administered 5 min after vehicle or ST1535 injection. Groups (III) and (IV) received benserazide (6 mg/kg i.p.) 30 min before L-DOPA injection. Contralateral rotations were measured for 10 min every 10 min for 2 h, while abnormal involuntary movements were measured for 1 min every 20 min for 2 h.

2.5. Evaluation of antitremorigenic effects of ST1535

To allow the observation of jaw tremors induced by tacrine, rats were individually placed in an elevated (40 cm from the bench) plexiglas cage with a metal grid over the floor; after habituation to the cage, they received an acute administration of ST1535 (10, 20 or 40 mg/kg i.p.) or its vehicle followed (5 min) by acute tacrine (2.5 mg/kg i.p.). Tacrine-induced jaw tremors were then recorded for 1 h subdivided into 6 tests of 10 min. The dose of tacrine used in this study was chosen to produce jaw tremors without inducing cholinergic peripheral stimulation, thus indicating that the tremor observed was originated by central, rather than peripheral, mechanisms (Salamone et al., 1998; Simola et al., 2004).

2.6. In situ hybridization

In situ hybridization studies were carried out three days after the end of subchronic treatment. Rats were killed with CO_2 and their brains were rapidly removed, frozen in dry ice-cooled isopentane, and stored at −20 °C. Cryostat coronal sections (12 μm) were mounted on glass slides coated with gelatine, and processed according to Carta et al. (2002). Sections were hybridized with [^{35}S]-labelled ribonucleotide probes complementary to mRNA encoding for GABA synthesizing enzyme glutamic acid decardoxylase (GAD67), dynorphin and enkephalin. Plasmids were linearized with SalI (Promega, Madison, WI, USA) (GAD67) and EcoRI (Promega) (dynorphin and enkephalin) restriction enzymes. Antisense ribonucleotide probes were generated using T3 RNA polymerase (Promega) (GAD67) and SP6 RNA polymerase (Promega) (dynorphin and enkephalin) in the presence of [^{35}S]UTP (Perkin Elmer, Milan, Italy). Each slide was hybridized with 100 μl of buffer containing 2×10^6 cpm of radioactively labelled probe. Hybridization was carried out at 55 °C overnight. The next morning slides were washed (1× SSC, room temperature; RNase A, 20 mg/ml, for 15 min; 4×20 min in 0.5× SSC at 60 °C, brief rinse in water), air-dried, and apposed to X-ray film.

2.7. Drugs

6-hydroxydopamine–HCl, desipramine, benserazide, L-DOPA and tacrine were purchased from Sigma-Aldrich (St. Louis, MO, USA). ST1535 (2-butyl-9-methyl-8-(2*H*-1,2,3-triazol-2-yl)-9*H*-purin-6-ylamine) was provided by Sigma–Tau (Rome, Italy). 6-hydroxydopamine–HCl, desipramine, benserazide, L-DOPA and tacrine were dissolved in saline, while ST1535 was dissolved in a solution formed by adding dimethylsulfoxide (DMSO, 10%), polyethylene glycol (PEG 400, 45%) and distilled water (45%). Drugs and vehicle were injected in a volume of 0.3 ml i.p. per 100 g body weight.

2.8. Data analysis and statistics

2.8.1. Statistic of behavioural studies

Mean and S.E.M. of the number of contralateral turns performed and mean and S.E.M. of seconds spent by rats in axial and limb abnormal involuntary movements in each 1 min testing-period were calculated throughout a 2 h period. Moreover, mean and S.E.M. of the number of jaw tremors were calculated for 10 min every 10 min for 1 h. Significance between groups was evaluated by two-way ANOVA followed by Newman–Keuls or Tukey's HSD post-hoc test. Results were considered significant at $P<0.05$.

2.8.2. Analysis and statistic of autoradiograms

One section from middle striatal levels for each rat (10.20 mm anterior to the interaural line; Paxinos and Watson, 1998) was examined for mRNA evaluation. Quantitative labelling analysis was performed using the image analysis program Scion Image. The average grey values from white matter were subtracted from each section to correct for background labelling. GAD67, dynorphin and enkephalin mRNA levels were measured in dorsolateral and ventromedial portions of the lesioned and unlesioned striatum, respectively. Effect of lesion and drug treatments on mRNA levels were determined by two-way ANOVA, followed by Tukey's HSD post-hoc test. Results were considered significant at $P<0.05$.

3. Results

3.1. Effect of acute ST1535 on L-DOPA-induced turning behaviour

6-hydroxydopamine lesioned rats were acutely administered with ST1535 at doses of 10, 20 or 40 mg/kg (i.p.) or vehicle (i.p.) plus a threshold dose of L-DOPA (3 mg/kg i.p.). ST1535 (20 and 40 mg/kg i.p.) significantly increased the number of contralateral turns induced by L-DOPA (3 mg/kg i.p.), as shown in Table 1. ST1535 (10 mg/kg i.p.) also increased the number of

Table 1
Effect of different doses of ST1535 on contralateral turning induced by L-DOPA

	Total contralateral turns in 2 h
ST1535 (10 mg)+L-DOPA (3 mg)	419±117.4
ST1535 (20 mg)+L-DOPA (3 mg)	747.4±196[a]
ST1535 (40 mg)+L-DOPA (3 mg)	638.6±107.9[a]
Vehicle+L-DOPA (3 mg)	129±37.1

Effect of acute administration of ST1535 (10, 20 and 40 mg) on contralateral turning induced by L-DOPA (3 mg/kg). ST1535 (20 and 40 mg/kg) significantly increased the number of contralateral turns induced by L-DOPA (3 mg/kg). [a]$P<0.05$; Newman–Keuls post-hoc test N=4-14.

contralateral turns but the difference with L-DOPA alone did not reach statistical difference.

Rats administered with the higher dose of ST1535 showed, in the first 20 min, flattened posture on the bottom of the hemispherical bowl; moreover, ST1535 induced weak homolateral turning behaviour during the 2 h observation period.

3.2. Effect of subchronic L-DOPA and ST1535+L-DOPA on turning behaviour and abnormal involuntary movements

On the basis of acute studies the dose of 20 mg/kg of ST1535 was chosen for further investigation in subchronic treatment.

6-hydroxydopamine lesioned rats were subchronically treated for 18 days with vehicle (i.p.), ST1535 (20 mg/kg i.p.), ST1535 (20 mg/kg i.p.)+L-DOPA (3 mg/kg i.p.) or with vehicle (i.p.)+L-DOPA (6 mg/kg i.p.). ST1535 (20 mg/kg i.p.)+L-DOPA (3 mg/kg i.p.) induced turning behaviour of similar intensity to L-DOPA (6 mg/kg i.p.) in the first administration.

Two-way ANOVA followed by Tukey's HSD post-hoc test showed a significant difference in the turning behaviour elicited by subchronic treatment with L-DOPA (6 mg/kg i.p.) on days 17 and 18 as compared to day 1. By contrast, subchronic treatment with ST1535+L-DOPA produced a turning behaviour which did not significantly differ during the course of the treatment (Fig. 1A).

Two-way ANOVA followed by Tukey's HSD post-hoc test showed a significant difference in the time spent by L-DOPA (6 mg/kg i.p.) subchronic treated rats in axial abnormal involuntary movements on days 12, 15, 17 and 18 with respect to day 1. By contrast, subchronic treatment with ST1535+L-DOPA produced low intensity axial abnormal involuntary movements with no significant differences being displayed during the course of the treatment (Fig. 1B).

Two-way ANOVA followed by Tukey's HSD post-hoc test showed a significant difference in the time spent by L-DOPA (6 mg/kg i.p.) subchronic treated rats in limb abnormal involuntary movements on days 12, 15, 17 and 18 with respect to day 1. By contrast, subchronic treatment with ST1535+L-DOPA produced low intensity limb abnormal involuntary movements not significantly different during the course of the treatment (Fig. 1C).

Rats subchronically treated with ST1535 (20 mg/kg i.p.) showed no episodes of contralateral turning or axial and limb abnormal involuntary movements during the course of treatment (data not shown).

Moreover, as shown by previous studies, rats subchronically treated with L-DOPA (3 mg/kg i.p.) showed a turning behaviour of weak intensity that was not significantly different during the course of the treatment (Morelli and Pinna, 2001) (data not shown).

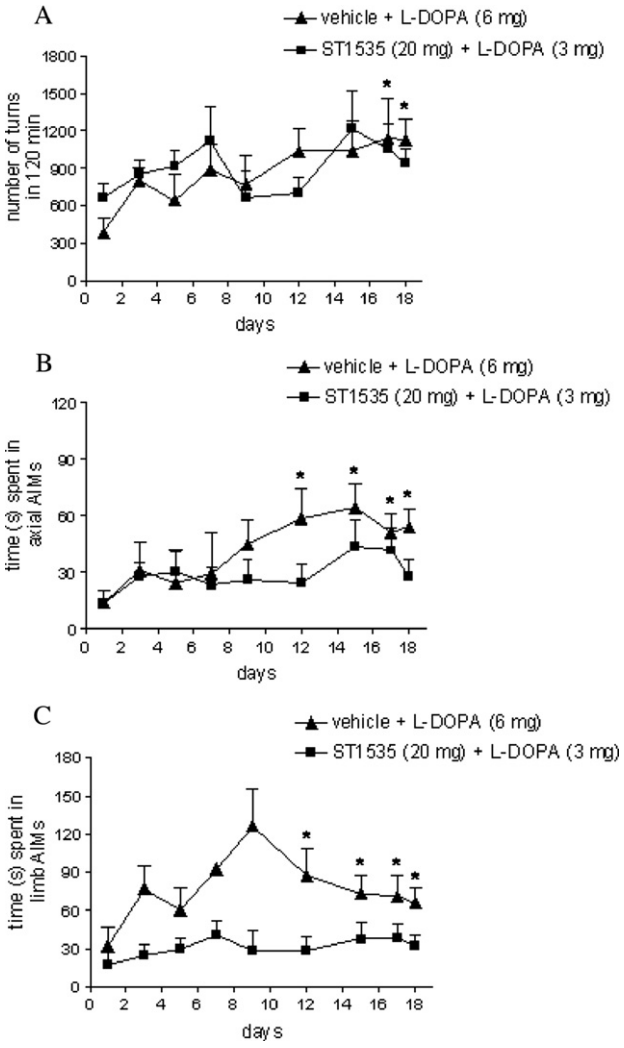

Fig. 1. Effect of subchronic L-DOPA (6 mg/kg) and ST1535 (20 mg/kg)+L-DOPA (3 mg/kg) on turning behaviour and abnormal involuntary movements. (A) Time–course analysis revealed a significant difference in the turning behavioural response to subchronic L-DOPA (6 mg/kg) from day 1 to days 17 and 18. *$P<0.05$; Tukey HSD post-hoc test. $N=6–8$. (B) Time–course analysis revealed a significant difference in the time spent by L-DOPA (6 mg/kg) subchronic treated rats in axial abnormal involuntary movements from day 1 to days 12, 15, 17 and 18. *$P<0.05$; Tukey HSD post-hoc test. $N=7–8$. (C) Time–course analysis revealed a significant difference in the time spent by L-DOPA (6 mg/kg) subchronic treated rats in limb abnormal involuntary movements from day 1 to days 12, 15, 17 and 18. *$P<0.05$; Tukey HSD post-hoc test. $N=7–8$.

3.3. Effect of ST1535 on tacrine-induced jaw tremors

Acute administration of ST1535 (20 mg/kg i.p.)+tacrine (2.5 mg/kg i.p.) induced a significant reduction of the number of jaw tremors as compared to vehicle+tacrine (2.5 mg/kg i.p.) (Fig. 2). Two-way ANOVA followed by Newman–Keuls post-hoc test revealed a significant reduction of jaw tremors in ST1535 (20 mg/kg, i.p.) treated rats at 20, 30 and 40 min after tacrine administration. Acute administration of ST1535 (10 mg/kg i.p.)+tacrine (2.5 mg/kg i.p.) did not significantly modify number of jaw tremors as compared to vehicle+tacrine (2.5 mg/kg i.p.). Number of jaw tremors: 1464±460 for ST1535 (10 mg/kg i.p.)+tacrine (2.5 mg/kg i.p.) and 1742±155 for vehicle+tacrine (2.5 mg/kg i.p.).

Acute administration of ST1535 (40 mg/kg i.p.)+tacrine (2.5 mg/kg i.p.) produced no further reduction in number of jaw tremors as compared to ST1535 (20 mg/kg i.p.)+tacrine (2.5 mg/kg i.p.) (data not shown).

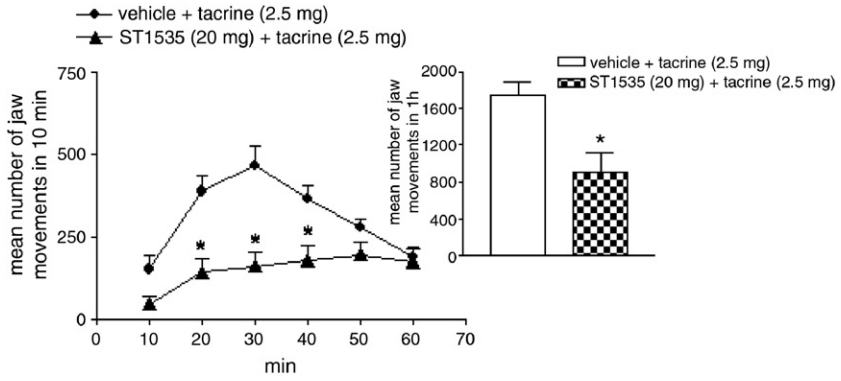

Fig. 2. Effect of acute ST1535 (20 mg/kg) on tacrine-induced jaw tremors. ST1535 (20 mg/kg) effectively counteracted jaw tremors stimulated by acute tacrine (2.5 mg/kg). Time point analysis revealed a significant effect at 20, 30 and 40 min after tacrine administration for ST1535 (20 mg/kg). *$P<0.05$ vs vehicle+tacrine; Newman–Keuls post-hoc test. $N=6–14$.

3.4. mRNA GAD67 levels after subchronic treatments

Two-way ANOVA followed by Tukey's HSD post-hoc test showed that 6-hydroxydopamine lesion significantly increased GAD67 mRNA levels in dorsolateral and ventromedial lesioned striatum as compared to unlesioned side (Fig. 3A–B). Subchronic treatment with L-DOPA (6 mg/kg i.p.) further increased GAD67 mRNA levels in the lesioned side as compared to lesioned side of vehicle subchronic treated rats, both in the dorsolateral and ventromedial striatum (Fig. 3A–B). In the lesioned side, GAD67 mRNA levels following subchronic treatment with L-DOPA (6 mg/kg i.p.) were significantly increased also compared to ST1535 (20 mg/kg i.p.) and ST1535 (20 mg/kg i.p.)+L-DOPA (3 mg/kg i.p.) in the dorsolateral striatum, and to ST1535 (20 mg/kg i.p.)+L-DOPA (3 mg/kg i.p.) in the ventromedial striatum (Fig. 3A–B). The

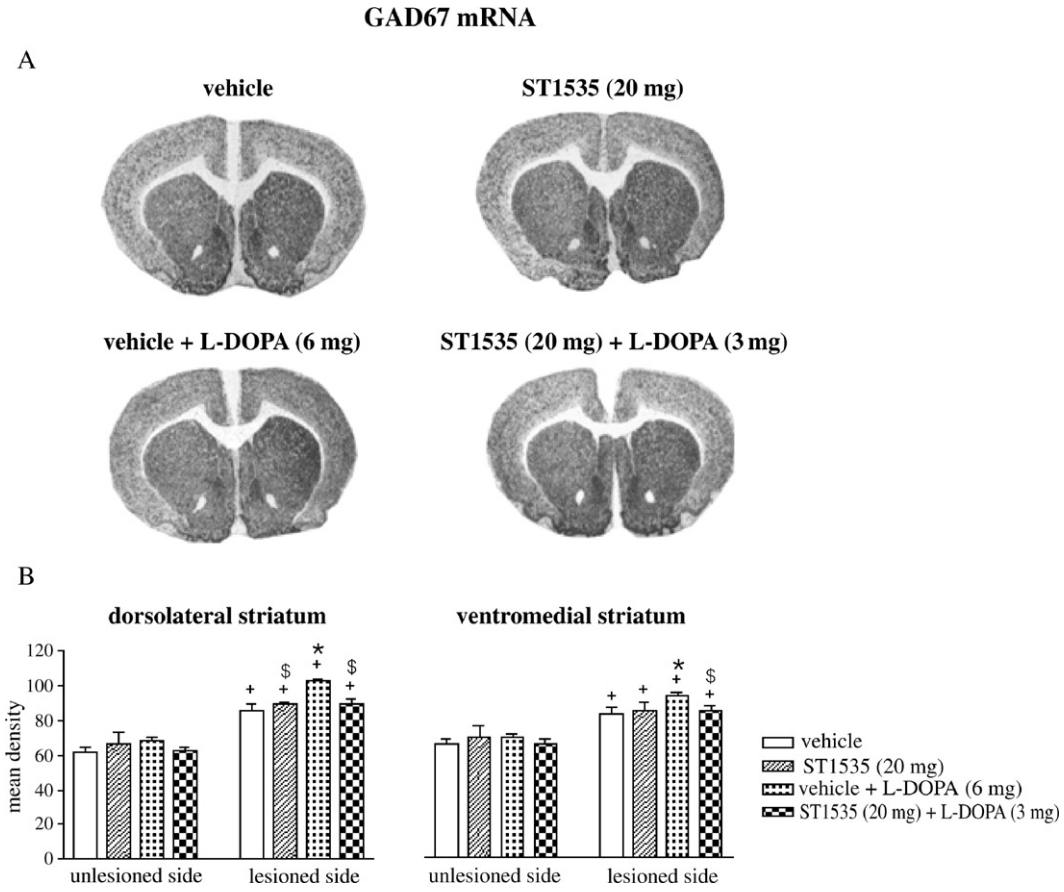

Fig. 3. (A) Autoradiograms showing *in situ* hybridization for GAD67 mRNA levels in the striatum of 6-hydroxydopamine lesioned rats subchronically treated with vehicle, ST1535 (20 mg/kg), L-DOPA (6 mg/kg) or ST1535 (20 mg/kg)+L-DOPA (3 mg/kg). (B) Mean density of GAD67 mRNA levels in unlesioned and lesioned dorsolateral and ventromedial striatum of rats subchronically treated as previously described. $^{+}P<0.05$ vs respective unlesioned side; *$P<0.05$ vs lesioned side of rats subchronically treated with vehicle; $^{\$}P<0.05$ vs lesioned side of rats subchronically treated with L-DOPA (6 mg/kg). $N=3–9$.

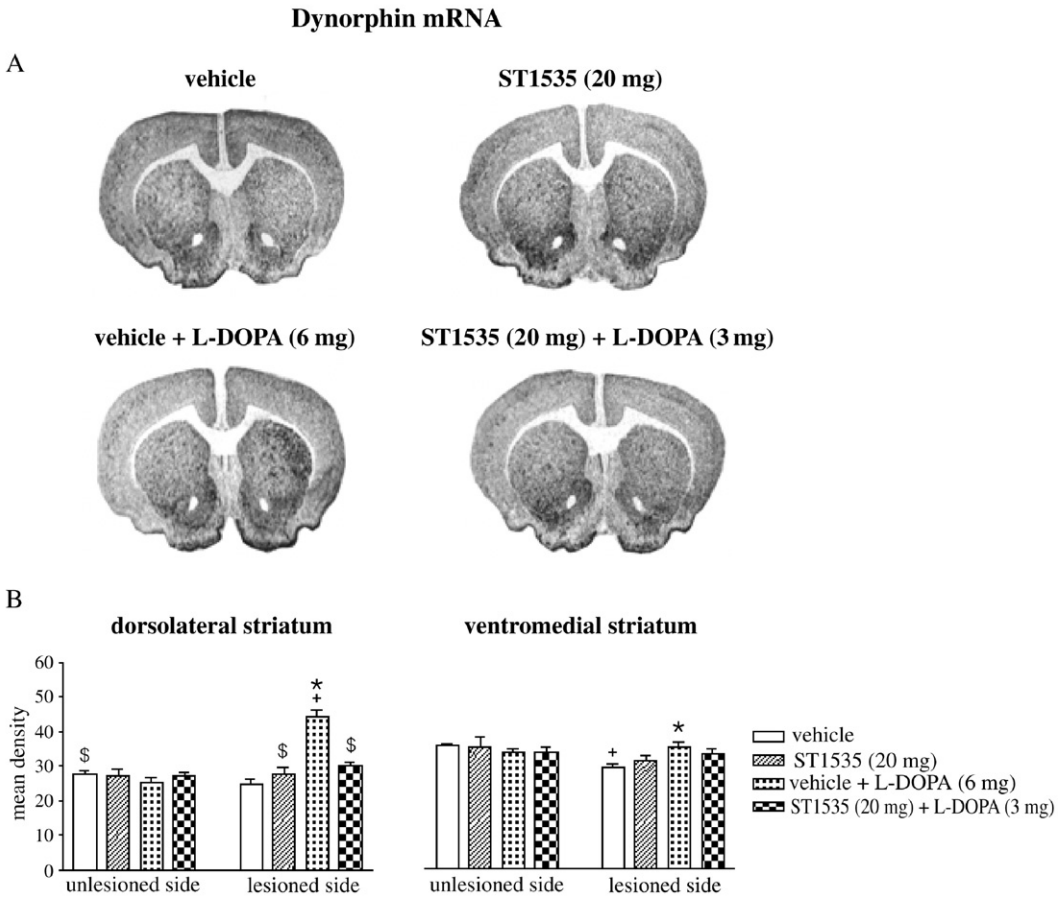

Fig. 4. (A) Autoradiograms showing *in situ* hybridization for dynorphin mRNA levels in the striatum of 6-hydroxydopamine lesioned rats subchronically treated with vehicle, ST1535 (20 mg/kg), L-DOPA (6 mg/kg) or ST1535 (20 mg/kg)+L-DOPA (3 mg/kg). (B) Mean density of dynorphin mRNA levels in unlesioned and lesioned dorsolateral and ventromedial striatum of rats subchronically treated as previously described. $^{+}P<0.05$ vs respective unlesioned side; $^{*}P<0.05$ vs lesioned side of rats subchronically treated with vehicle; $^{\$}P<0.05$ vs lesioned side of rats subchronically treated with L-DOPA (6 mg/kg). $N=3$–9.

levels of GAD67 mRNA in the unlesioned side of striatum did not differ significantly among the experimental groups. As shown by previous studies, subchronic treatment with L-DOPA (3 mg/kg i.p.) did not modify GAD67 mRNA striatal expression (Carta et al., 2002) (data not shown).

3.5. mRNA dynorphin levels after subchronic treatments

Two-way ANOVA followed by Tukey's HSD post-hoc test showed that 6-hydroxydopamine lesion significantly decreased dynorphin mRNA levels in the lesioned ventromedial striatum as compared to unlesioned side (Fig. 4A–B). Subchronic treatment with L-DOPA (6 mg/kg i.p.) increased dynorphin mRNA levels in the lesioned dorsolateral striatum as compared to lesioned and unlesioned side of vehicle subchronic treated rats and to the respective unlesioned side (Fig. 4A–B). Subchronic L-DOPA (6 mg/kg i.p.) increased dynorphin mRNA levels in the ventromedial striatum as compared to lesioned but not to unlesioned side of vehicle treated rats (Fig. 4A–B). In the lesioned dorsolateral striatum, after subchronic L-DOPA (6 mg/kg i.p.), dynorphin mRNA levels were significantly increased compared to ST1535 (20 mg/kg i.p.) and ST1535 (20 mg/kg i.p.)+L-DOPA (3 mg/kg i.p.) (Fig. 4A–B). The levels of dynorphin mRNA in the unlesioned side of striatum did not differ significantly among the experimental groups. As shown by previous studies, subchronic treatment with L-DOPA (3 mg/kg i.p.) did not modify dynorphin mRNA striatal expression (Carta et al., 2002) (data not shown).

3.6. mRNA enkephalin levels after subchronic treatment

Two-way ANOVA followed by Tukey's HSD post-hoc test showed that 6-hydroxydopamine lesion significantly increased enkephalin mRNA levels in the lesioned dorsolateral and ventromedial striatum as compared to unlesioned side (Fig. 5A–B). Subchronic treatment with L-DOPA (6 mg/kg i.p.) further increased enkephalin mRNA levels in the lesioned dorsolateral striatum as compared to lesioned side of vehicle subchronic treated rats (Fig. 5A–B). All treatments showed higher enkephalin mRNA levels in the unlesioned dorsolateral and ventromedial striatum as compared to unlesioned side of vehicle treated rats, with the exception of ST1535 (20 mg/kg i.p.) in which a similar effect could be observed only in the dorsolateral striatum (Fig. 5A–B). As shown by previous studies,

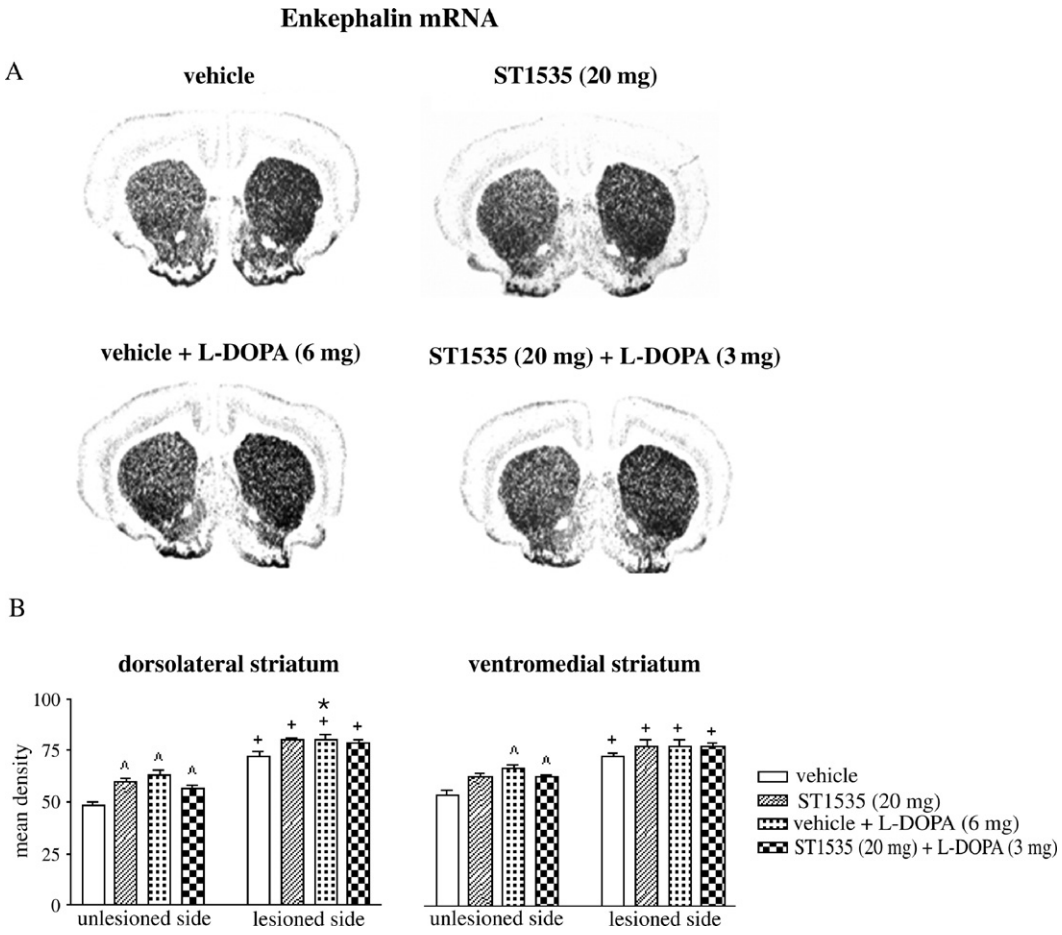

Fig. 5. (A) Autoradiograms showing *in situ* hybridization for enkephalin mRNA levels in the striatum of 6-hydroxydopamine lesioned rats subchronically treated with vehicle, ST1535 (20 mg/kg), L-DOPA (6 mg/kg) or ST1535 (20 mg/kg)+L-DOPA (3 mg/kg). (B) Mean density of enkephalin mRNA levels in unlesioned and lesioned dorsolateral and ventromedial striatum of rats subchronically treated as previously described. $^{+}P<0.05$ vs respective unlesioned side; $*P<0.05$ vs lesioned side of rats subchronically treated with vehicle; $\wedge P<0.05$ vs unlesioned side of rats subchronically treated with vehicle. $N=3–9$.

subchronic treatment with L-DOPA (3 mg/kg i.p.) did not modify enkephalin mRNA striatal expression (Carta et al., 2002) (data not shown).

4. Discussion

In view of the promising antiparkinsonian properties of adenosine A_{2A} receptor antagonists reported by several authors (Grondin et al., 1999; Kanda et al., 2000; Pinna et al., 2001; Xu et al., 2005) and with the aim of obtaining novel efficient drugs, considerable attention is currently being paid to the synthesis of new adenosine A_{2A} receptor antagonists and their pre-clinical evaluation. The present study evaluated motor and biochemical effects of the new adenosine A_{2A} receptor antagonist ST1535 in the 6-hydroxydopamine rat model of Parkinson's disease, in order to verify whether the property of ST1535 to antagonize "*in vitro*" adenosine A_{2A} receptors resulted in functional antagonistic actions.

The results obtained showed that, similar to other adenosine A_{2A} receptor antagonists, ST1535 produces positive effects in acute model of parkinsonian akinesia and tremor as well as in subchronic models of dyskinesia evaluating long-term modifications of drug effects.

Adenosine A_{2A} receptor antagonistic properties of ST1535 have been shown by previous studies demonstrating the higher affinity of the drug for A_{2A} than A_1 adenosine receptor subtype, its ability to inhibit cAMP synthesis induced by the adenosine A_{2A} receptor agonist NECA and its capacity to block catalepsy induced by the adenosine A_{2A} receptor agonist CGS21680 (Minetti et al., 2005; Stasi et al., 2006).

In the 6-hydroxydopamine rat model of Parkinson's disease, adenosine A_{2A} receptor antagonists increased turning behaviour induced by L-DOPA or by direct dopamine receptor agonists, showing antiparkinsonian effects (Fenu et al., 1997; Jenner, 2003; Jiang et al., 1993; Koga et al., 2000). In line with these results, and as shown by the present and previous studies, ST1535 increased, at doses similar to other adenosine A_{2A} receptor antagonists, the contralateral turning induced by L-DOPA in rats, whereas in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treated primates the compound improved locomotor activity and decreased motor disability when given in association to a low dose of L-DOPA (Rose et al., 2006, 2007).

When administered subchronically with a threshold dose of L-DOPA, ST1535 induced a similar intensity of contralateral turning to that produced by a full dose of L-DOPA; however, during the course of the treatment this drug combination did not

induce sensitization to contralateral turning behaviour and abnormal involuntary movements. Although compared to L-DOPA alone ST1535+L-DOPA treatment exerted a significantly greater prevention on abnormal involuntary movements than on contralateral turning behaviour, the results obtained clearly demonstrate the low dyskinetic potential of ST1535+L-DOPA. Therefore, administration of ST1535, by potentiating the effect of L-DOPA, induces motor effects similar to those induced by a higher dose of L-DOPA without exacerbating the dyskinetic effects.

The results obtained in this study are in agreement with previous studies showing that repeated blockade of adenosine A_{2A} receptors+L-DOPA displays low dyskinetic-like side effects (Bibbiani et al., 2003; Grondin et al., 1999; Pinna et al., 2001).

Consistent studies performed in 6-hydroxydopamine lesioned rats indicate that sensitization to turning behaviour and abnormal involuntary movements induced by intermittent L-DOPA treatment is associated to alteration of neuronal activity in the lesioned striatum. Increase of GAD67 and dynorphin mRNA expression showed a correlation between their adaptative modifications in output striatal neurons and L-DOPA-induced dyskinesia (Carta et al., 2002; Cenci et al., 1998; Henry et al., 2003; Nielsen and Soghomonian, 2004; Soghomonian et al., 1996; Zeng et al., 1995).

A previous study performed by our group reported long-term changes in GAD67, dynorphin and enkephalin mRNA levels after subchronic L-DOPA but not after adenosine A_{2A} receptor antagonists+L-DOPA treatment (Carta et al., 2002). In agreement with the previous study, the results of the present work show a marked increase of mRNA levels for GAD67 and dynorphin, and a slight, although significant increase of mRNA for enkephalin in dorsolateral 6-hydroxydopamine lesioned striatum following subchronic L-DOPA treatment. On the contrary, rats subchronically treated with ST1535+L-DOPA and ST1535 alone showed no or low modifications in levels of mRNA for these markers. Moreover, these rats displayed significantly lower GAD67 and dynorphin mRNA levels than those of L-DOPA subchronic treated rats.

The lack of modifications on mRNA expression produced in striatal neuronal markers might suggest that repeated coadministration of ST1535+L-DOPA did not induce adaptative changes on striatal neuronal activity, which in turn may explain the stable turning behavioural response and the low intensity of abnormal involuntary movements observed during ST1535+L-DOPA treatment.

Therefore, association between ST1535+L-DOPA, in contrast to observations made with repeated intermittent administration of a fully effective dose of L-DOPA, might be capable of producing antiakinetic effects without excessive activation of striatal efferent neurons. This finding would seem to suggest the ability of ST1535 to increase the therapeutic efficacy of L-DOPA while maintaining the side effects elicited by L-DOPA to levels associated to low doses of L-DOPA. Selective localization of adenosine A_{2A} receptors in striatal region and their direct interaction at receptor and transcriptional level with dopamine D_2 receptors, together with indirect interaction with dopamine D_1 receptors at basal ganglia level, might underlie the effects observed in this study (Pinna et al., 1996; Ferré et al., 1997; Le Moine et al., 1997). Therefore, ST1535, by blocking adenosine A_{2A} receptor, potentiates dopamine transmission, allowing administration of a low dose of L-DOPA and reducing neuronal long-term modifications correlated to L-DOPA motor side effects.

In addition to the efficacy demonstrated in restoring motor behaviour in models of Parkinson's disease without inducing dyskinetic-like effects, adenosine A_{2A} receptor antagonists produce positive effects on parkinsonian tremor (Correa et al., 2004; Simola et al., 2004).

Using the rat model of tacrine-induced jaw tremors, as index of parkinsonian tremor (Salamone et al., 1998), we observed that acute ST1535 was able to significantly reduce these jaw tremors. The antitremorigenic effect is most probably exerted through an action of the compound on adenosine A_{2A} receptors localized on striatal cholinergic nerve terminals controlling the acetylcholine levels in striatum (Brown et al., 1990; Kurokawa et al., 1996). Thus, similar to observations made with different adenosine A_{2A} receptor antagonists (Chase et al., 2003; Correa et al., 2004; Simola et al., 2004), blockade of adenosine A_{2A} receptors by ST1535 may be capable of attenuating the increased cholinergic tone mediated by tacrine and of explicating an antitremorigenic activity.

ST1535 is a recently developed adenosine A_{2A} receptor antagonist displaying marked antiparkinsonian activity in 6-hydroxydopamine lesioned rats and antitremorigenic effects in tacrine-treated rats. The positive effects reported following subchronic administration are not associated to long-term modifications in behavioural responses and in neuronal activity in the 6-hydroxydopamine lesioned striatum, thus emphasising the potential use of ST1535 as a novel therapeutic approach in Parkinson's disease.

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References

Bara-Jimenez, W., Sherzai, A., Dimitrova, T., Favit, A., Bibbiani, F., Gillespie, M., Morris, M.J., Mouradian, M.M., Chase, T.N., 2003. Adenosine A(2A) receptor antagonist treatment of Parkinson's disease. Neurology 61, 293–296.

Bibbiani, F., Oh, J.D., Petzer, J.P., Castagnoli, N., Chen Jr., J.F., Schwarzschild, M.A., Chase, T.N., 2003. A2A antagonist prevents dopamine agonist-induced motor complications in animal models of Parkinson's disease. Exp. Neurol. 184, 285–294.

Brown, S.J., James, S., Reddington, M., Richardson, P.J., 1990. Both A1 and A2a purine receptors regulate striatal acetylcholine release. J. Neurochem. 55, 31–38.

Carta, A.R., Pinna, A., Cauli, O., Morelli, M., 2002. Differential regulation of GAD67, enkephalin and dynorphin mRNAs by chronic-intermittent L-dopa and A2A receptor blockade plus L-dopa in dopamine-denervated rats. Synapse 44, 166–174.

Carta, A.R., Pinna, A., Morelli, M., 2006. How reliable is the behavioural evaluation of dyskinesia in animal models of Parkinson's disease? Behav. Pharmacol. 17, 393–402.

Cenci, M.A., Lee, C.S., Bjorklund, A., 1998. L-DOPA-induced dyskinesia in the rat is associated with striatal overexpression of prodynorphin- and glutamic acid decarboxylase mRNA. Eur. J. Neurosci. 10, 2694–2706.

Chase, T.N., Bibbiani, F., Bara-Jimenez, W., Dimitrova, T., Oh-Lee, J.D., 2003. Translating A2A antagonist KW6002 from animal models to parkinsonian patients. Neurology 61, S107–S111.

Correa, M., Wisniecki, A., Betz, A., Dobson, D.R., O'Neill, M.F., O'Neill, M.J., Salamone, J.D., 2004. The adenosine A2A antagonist KF17837 reverses the locomotor suppression and tremulous jaw movements induced by haloperidol in rats: possible relevance to parkinsonism. Behav. Brain Res. 148, 47–54.

Delfino, M.A., Stefano, A.V., Ferrario, J.E., Taravini, I.R.E., Murer, M.G., Gershanik, O.S., 2004. Behavioral sensitization to different dopamine agonists in a parkinsonian rodent model of drug-induced dyskinesias. Behav. Brain Res. 152, 297–306.

Fenu, S., Pinna, A., Ongini, E., Morelli, M., 1997. Adenosine A_{2A} receptor antagonism potentiates L-DOPA induced turning behaviour and c-fos expression in 6-hydroxydopamine-lesioned rats. Eur. J. Pharmacol. 321, 143–147.

Ferré, S., Fredholm, B.B., Morelli, M., Popoli, P., Fuxe, K., 1997. Adenosine–dopamine receptor–receptor interactions as an integrative mechanism in the basal ganglia. Trends Neurosci. 20, 482–487.

Grondin, R., Bedard, P.J., Hadj Tahar, A., Gregoire, L., Mori, A., Kase, H., 1999. Antiparkinsonian effect of a new selective adenosine A_{2A} receptor antagonist in MPTP-treated monkeys. Neurology 52, 1673–1677.

Hauser, R.A., Hubble, J.P., Truong, D.D., Istradefylline US-001 Study Group, 2003. Randomized trial of the adenosine A(2A) receptor antagonist istradefylline in advanced PD. Neurology 61, 297–303.

Henry, B., Crossman, A.R., Brotchie, J.M., 1998. Characterization of enhanced behavioral responses to L-DOPA following repeated administration in the 6-hydroxydopamine-lesioned rat model of Parkinson's disease. Exp. Neurol. 151, 334–342.

Henry, B., Crossman, A.R., Brotchie, J.M., 1999. Effect of repeated L-DOPA, bromocriptine, or lisuride administration on preproenkephalin-A and preproenkephalin-B mRNA levels in the striatum of the 6-hydroxydopamine-lesioned rat. Exp. Neurol. 155, 204–220.

Henry, B., Duty, S., Fox, S.H., Crossman, A.R., Brotchie, J.M., 2003. Increased striatal pre-proenkephalin B expression is associated with dyskinesia in Parkinson's disease. Exp. Neurol. 183, 458–468.

Jenner, P., 2003. A2A antagonists as novel non-dopaminergic therapy for motor dysfunction in PD. Neurology 61, S32–S38.

Jiang, H., Jackson-Lewis, V., Muthane, U., Dollison, A., Ferreira, M., Espinosa, A., Parsons, B., Przedborski, S., 1993. Adenosine receptor antagonists potentiate dopamine receptor agonist-induced rotational behavior in 6-hydroxydopamine-lesioned rats. Brain Res. 613, 347–351.

Kanda, T., Jackson, M.J., Smith, L.A., Pearce, R.K., Nakamura, J., Kase, H., Kuwana, Y., Jenner, P., 2000. Combined use of the adenosine A(2A) antagonist KW-6002 with L-DOPA or with selective D1 or D2 dopamine agonists increases antiparkinsonian activity but not dyskinesia in MPTP-treated monkeys. Exp. Neurol. 162, 321–327.

Koga, K., Kurokawa, M., Ochi, M., Nakamura, J., Kuwana, Y., 2000. Adenosine A(2A) receptor antagonists KF17837 and KW-6002 potentiate rotation induced by dopaminergic drugs in hemi-Parkinsonian rats. Eur. J. Pharmacol. 408, 249–255.

Kurokawa, M., Koga, K., Kase, H., Nakamura, J., Kuwana, Y., 1996. Adenosine A2A receptor-mediated modulation of striatal acetylcholine release in vivo. J. Neurochem. 66, 1882–1888.

Le Moine, C., Svenningsson, P., Fredholm, B.B., Bloch, B., 1997. Dopamine–adenosine interactions in the striatum and the globus pallidus: inhibition of striatopallidal neurons through either D_2 or A_{2A} receptors enhances D1 receptor-mediated effects on c-fos expression. J. Neurosci. 15, 8038–8048.

Lundblad, M., Andersson, M., Winkler, C., Kirik, D., Wierup, N., Cenci, M.A., 2002. Pharmacological validation of behavioural measures of akinesia and dyskinesia in a rat model of Parkinson's disease. Eur. J. Neurosci. 15, 120–132.

Minetti, P., Tinti, M.O., Carminati, P., Castorina, M., Di Cesare, M.A., Di Serio, S., Gallo, G., Ghirardi, O., Giorni, F., Giorgi, L., Piersanti, G., Bartoccini, F., Tarzia, G., 2005. 2-*n*-Butyl-9-methyl-8-[1,2,3]triazol-2-yl-9*H*-purin-6-ylamine and analogues as A2A adenosine receptor antagonists. Design, synthesis, and pharmacological characterization. J. Med. Chem. 48, 6887–6896.

Morelli, M., 2003. Adenosine A2A antagonists: potential preventive and palliative treatment for Parkinson's disease. Exp. Neurol. 184, 20–23.

Morelli, M., Pinna, A., 2001. Interaction between dopamine and adenosine A2A receptors as a basis for the treatment of Parkinson's disease. Neurol. Sci. 22, 71–72.

Nielsen, K.M., Soghomonian, J.J., 2004. Normalization of glutamate decarboxylase gene expression in the entopeduncolar nucleus of rats with a unilateral 6-hydroxydopamine lesion correlates with increased GABAergic input following intermittent but not continuous levodopa. Neuroscience 123, 31–42.

Paxinos, G., Watson, C., 1998. The Rat Brain in Stereotaxic Coordinates, 4th edn. Academic Press, San Diego, USA.

Pellegrino, L.J., Pellegrino, A.S., Cushman, A.J., 1979. A Stereotaxic Atlas of the Rat Brain. Plenum Press, New York, NY.

Pinna, A., Di Chiara, G., Wardas, J., Morelli, M., 1996. Blockade of A2a adenosine receptors positively modulates turning behaviour and c-Fos expression induced by D1 agonists in dopamine-denervated rats. Eur. J. Neurosci. 8, 1176–1181.

Pinna, A., Fenu, S., Morelli, M., 2001. Motor stimulant effects of the adenosine A2A receptor antagonist SCH 58261 do not develop tolerance after repeated treatment in 6-hydroxydopamine-lesioned rats. Synapse 39, 233–238.

Pinna, A., Pontis, S., Morelli, M., 2006. Expression of dyskinetic movements and turning behaviour in subchronic l-DOPA 6-hydroxydopamine-treated rats is influenced by the testing environment. Behav. Brain Res. 171, 175–178.

Ravenscroft, P., Chalon, S., Brotchie, J.M., Crossman, A.R., 2004. Ropinirole versus L-DOPA effects on striatal opioid peptide precursor in a rodent model of Parkinson's disease: implications for dyskinesia. Exp. Neurol. 185, 36–46.

Rose, S., Jackson, M.J., Smith, L.A., Stockwell, K., Johnson, L., Carminati, P., Jenner, P., 2006. The novel adenosine A_{2a} antagonist ST1535 potentiates the effects of a threshold dose of L-DOPA in MPTP treated common marmosets. Eur. J. Pharmacol. 546, 82–87.

Rose, S., Ramsay Croft, N., Jenner, P., 2007. The novel adenosine A_{2A} antagonist ST1535 potentiates the effects of a threshold dose of L-DOPA in unilaterally 6-hydroxydopamine lesioned rats. Brain Res. 1133, 110–114.

Salamone, J.D., Mayorga, A.J., Trevitt, J.T., Cousins, M.S., Conlan, A., Nawab, A., 1998. Tremulous jaw movements in rats: a model of parkinsonian tremor. Prog. Neurobiol. 56, 591–611.

Schwarzschild, M.A., Agnati, L., Fuxe, K., Chen, J.F., Morelli, M., 2006. Targeting adenosine A(2A) receptors in Parkinson's disease. Trends Neurosci. 29, 647–654.

Simola, N., Fenu, S., Baraldi, P.G., Tabrizi, M.A., Morelli, M., 2004. Blockade of adenosine A_{2A} receptors antagonizes parkinsonian tremor in the rat tacrine model by an action on specific striatal regions. Exp. Neurol. 189, 182–188.

Soghomonian, J.J., Pedneault, S., Blanchet, P.J., Goulet, M., Di Paolo, T., Bedard, P.J., 1996. L-DOPA regulates glutamate decarboxylases mRNA levels in MPTP-treated monkeys. Mol. Brain Res. 39, 237–240.

Stasi, M.A., Borsini, F., Varani, K., Vincenzi, F., Di Cesare, M.A., Minetti, P., Ghirardi, O., Carminati, P., 2006. ST1535: a preferential A2A adenosine receptor antagonist. Int. J. Neuropsychopharmacol. 9, 575–584.

Xu, K., Bastia, E., Schwarzchild, M., 2005. Therapeutic potential of adenosine A(2A) receptor antagonist in Parkinson's disease. Pharmacol. Ther. 105, 267–310.

Zeng, B.Y., Jolkkonen, J., Jenner, P., Marsden, C.D., 1995. Chronic L-DOPA treatment differentially regulates gene expression of glutamate decarboxylase, preproenkephalin and preprotachykinin in the striatum of 6-hydroxydopamine lesioned rat. Neuroscience 66, 19–28.