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Research Report

Inhibitory effect of 8-(3-chlorostryryl) caffeine on levodopa-induced motor fluctuation is associated with intracellular signaling pathway in 6-OHDA-lesioned rats

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ABSTRACT

8-(3-chlorostryryl) caffeine (CSC), a selective adenosine A_{2A} receptor antagonist, has been reported to inhibit the levodopa-induced motor fluctuation in Parkinson's disease. However, the underlying mechanism of its action remains largely unknown. In our study, we investigated the signaling pathway by which CSC inhibited levodopa-induced motor fluctuation in rats with a 6-hydroxydopamine (6-OHDA)-induced lesion. We treated 6-OHDA-lesioned rats with levodopa (50 mg/kg/day, twice daily) for 22 days, followed by levodopa + CSC (5 mg/kg/day, twice daily) or levodopa + vehicle for 7 days. The sham-lesioned and 6-OHDA-lesioned rats treated with saline for 29 days served as sham and lesion control groups. We found that the treatment of CSC reversed the shortening of the rotational motor response duration induced by levodopa administration and the effect was maintained until the end of the treatment. The chronic levodopa treatment upregulated the adenosine A_{2A} receptor expression and modified downstream signaling pathway including decreasing the phosphorylation of DARPP-32 at Thr75 site and increasing the phosphorylation of ERK1/2 in the lesioned striatum. However, the following CSC treatment attenuated the levodopa-induced adenosine A_{2A} receptor upregulation and abolished the aberrant phosphorylation of DARPP-32 at Thr75 site and that of ERK1/2. Our results indicate that the inhibitory effect of CSC on levodopa-induced motor fluctuation may be associated with the inhibition of Adenosine A_{2A} Receptor and downstream DARPP-32 and ERK1/2 signaling pathway.

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder of the central nervous system, which is manifested by a loss of the dopaminergic nigrostriatal pathway as part of its core pathology. Consequently, replacement of this lost network pharmacologically with dopamine represents the most effective drug therapy for the symptomatic relief in PD. While initially successful in alleviating the motor symptoms of PD, long-term therapy, particularly involving levodopa, is associated with various motor complications, which are difficult to control. Recently, the idea has gone mainstream that non-dopaminergic systems may play major roles in PD (Lang and Obeso, 2004).

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Adenosine A_{2A} receptor is one of the potential drug targets for PD treatment. Adenosine A_{2A} receptor is selectively localized in the medium spiny neurons that form the indirect pathway from the striatum to the lateral globus pallidus containing GABA and enkephalin (Schiffmann and Vanderhaeghen, 1993; Svenningsson et al., 1997). This receptor appears to contribute to the induction of levodopa-induced behavioral alterations (Fredduzzi et al., 2002) and its antagonist is reported to decrease the propensity for levodopa-induced dyskinesia (LID) (Grondin et al., 1999; Kanda et al., 2000). However, the intracellular mechanism underlying these effects is largely unknown.

Dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) are two major striatal signaling pathway components. Both of them are involved in the development of long-term changes of gene expression, synaptic plasticity, and locomotor responses (Valjent et al., 2000, 2004; Berhow et al., 1996). Recent studies confirmed that DARPP-32 and ERK1/2 undergo modification in the pathological progress of PD and levodopa-induced motor complications.

DARPP-32 is selectively enriched in medium spiny neurons receiving dopaminergic input (Ouimet et al., 1998). Its activity depends on the two phosphorylation sites: Thr34 and Thr75. Phosphorylation of DARPP-32 at Thr34 by cAMP-dependent protein kinase (PKA) results in its conversion into a potent inhibitor of protein phosphatase 1 (PP1), amplifying the effect of the PKA pathway. Phosphorylation at the Thr75 by cyclin-dependent kinase 5 (Cdk5) inhibits PKA activity (Bibb et al., 1999). Therefore different phosphorylation states act in the opposite way and control many downstream effectors including neurotransmitter receptors and voltage-gated ion channels (Greengard et al., 1999).

ERK1/2 belongs to the mitogen-activated protein kinase (MAPK) family. The phosphorylation state of ERK1/2 reflects a balance between the activities of upstream kinases and inactivating phosphatases (Keyse, 2000). Active ERK1/2 mediates changes in gene expression (Deak et al., 1998; Dunn et al., 2005) and activation of nuclear transcription factors, including Elk-1 and CREB (Marshall, 1995).The possible involvement of ERK pathway in levodopa-mediated motor disturbances is suggested by recent studies showing that, in 6-hydroxydopamine-lesioned mice, chronic administration of levodopa is accompanied by enhanced levels of phosphorylated ERK (Pavon et al., 2006).

Adenosine A_{2A} receptor agonist is shown to increase the DARPP-32 phosphorylation at Thr34 in striatal slices. Furthermore, it is reported that DARPP-32 is involved in the psychostimulant effect of A_{2A} antagonist. Recent work has demonstrated the importance of phospho-Thr34-DARPP-32 in the regulation of ERK1/2 (Valjent et al., 2005).It is also reported that adenosine A_{2A} receptor activation leads to the activation of ERK1/2 (Arslan and Fredholm, 2000; Wyatt et al., 2002) and ERK1/2 is implicated in the A_{2A} receptor mediated cell survival pathway. However, there have been very few reports about whether the two proteins are involved in the locomotor effects of A_{2A} antagonist in levodopa-induced motor complication.

Therefore, we studied the effects of A_{2A} antagonist CSC in 6-OHDA-lesioned hemiparkinsonian rats that developed response alterations induced by chronic twice-daily levodopa treatment to address the involvement of adenosine A_{2A} receptor in the levodopa-induced motor complication and explore the possible intracellular mechanism. Specifically, parkinsonian rats were assessed for the ability of A_{2A} receptor blockade to ameliorate the motoric changes associated with dopaminergic denervation and dopaminomimetic treatment in relation to striatal expression of adenosine A_{2A} receptor and phosphorylation of DARPP-32 and ERK1/2.

2. Results

2.1. CSC attenuated the reduction of rotational duration induced by levodopa

We first conducted series of experiments to access the behavioral effects of levodopa and CSC on pharmacologically treated animals. We found that levodopa, given twice daily for 22 days, significantly reduced the animals' response in a rotational motor task ($P<0.05$). The duration of the motor response decreased from 175±12.40 min to 120±12.12 min during the 22-day injection period (Fig. 1). The decrease of motor duration was maintained throughout the following seven days in the levodopa plus vehicle-treated group. Interestingly, on day 23 of levodopa treatment, the administration of CSC prior to the levodopa challenge significantly reversed the levodopa-induced reduction in the rotational duration ($P<0.05$). This effect was maintained until the end of the CSC subacute treatment and restored the rotational duration to the level of day 1.

2.2. CSC antagonized the effect of levodopa on striatal adenosine A_{2A} receptor upregulation

In order to understand the molecular mechanism underlying the behavioral changes induced by the pharmacological treatments shown above, we conducted biochemical tests in

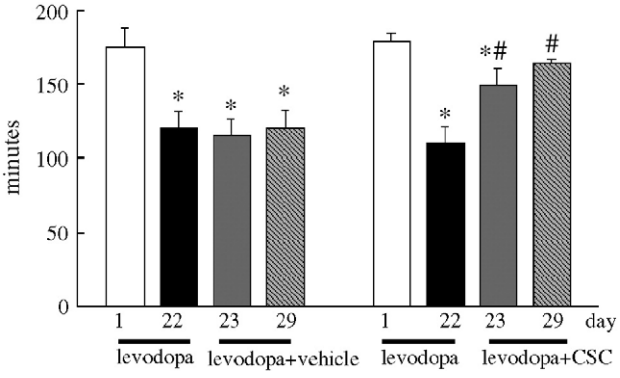

Fig. 1 – Effect of subacute administration of CSC on levodopa-induced shortening of the rotational motor response duration. 22 days of levodopa treatment reduced the rotational duration. On day 23, the subacute administration of levodopa+vehicle or levodopa+CSC was started for one additional week. The decrease of rotational duration was reversed by levodopa+CSC administration, while maintained by levodopa+vehicle administration. *$P<0.05$ and #$P<0.05$ versus day1 and day 22, respectively.

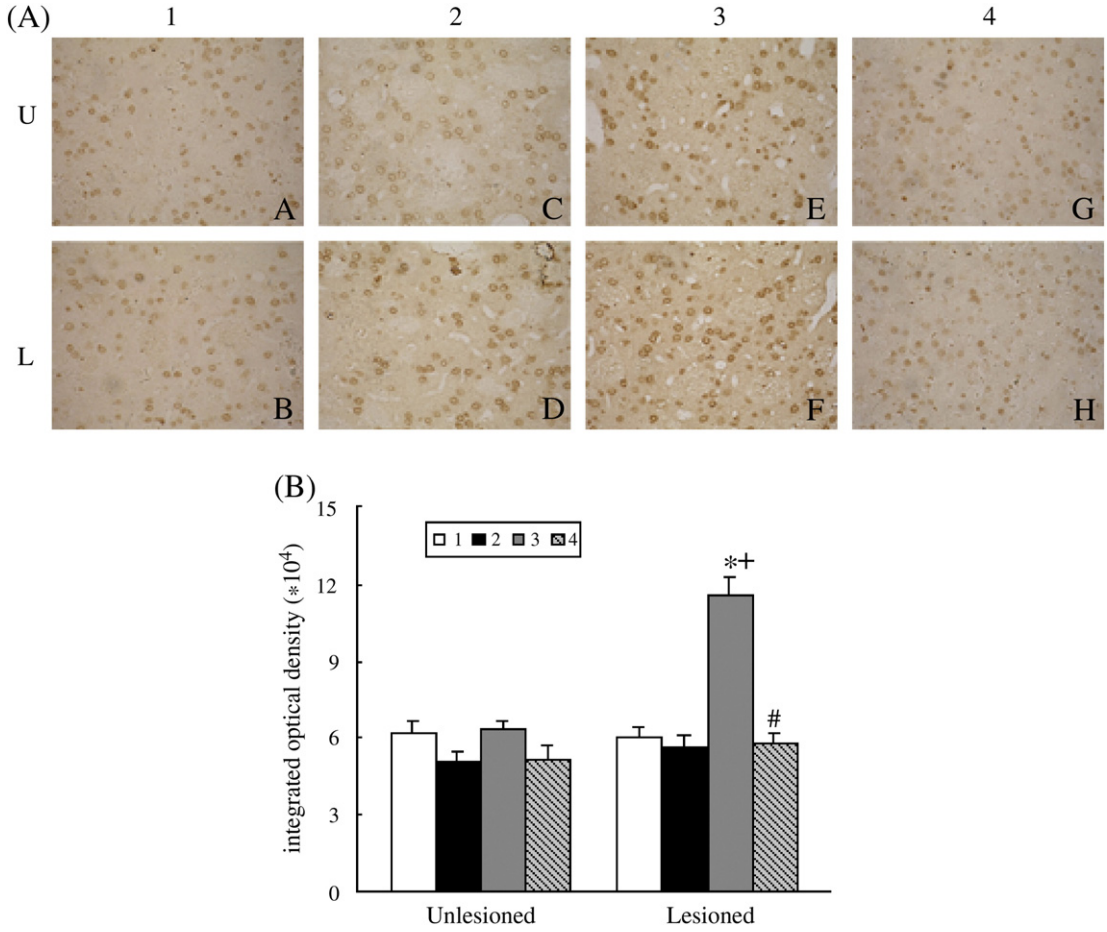

Fig. 2 – Effect of subacute administration of CSC on the levodopa-induced changes in striatal adenosine A_{2A} receptor expression. The striatal sections were analyzed by immunohistochemistry with antibodies specific for the A_{2A} receptor from sham (1), 6-OHDA-lesioned (2), 6-OHDA-lesioned plus levodopa treated (3), and 6-OHDA-lesioned plus levodopa in combination with CSC-treated (4) rats. U = unlesioned striatum, L = lesioned striatum. $^{*}P<0.05$ and $^{\#}P<0.05$ compared with 6-OHDA-lesioned, and 6-OHDA-lesioned plus levodopa rats, respectively. $^{+}P<0.05$ compared with the unlesioned striatum of the same group.

the brains of drug-treated and control animals. Immunohistochemistry tests revealed that, the adenosine A_{2A} receptor was predominantly located in the cell membrane (Fig. 2). 6-OHDA lesion did not alter the level of striatal A_{2A} receptor expression, as no statistically significant difference was detected between the lesioned side and either the unlesioned side or the sham-lesioned group. However, chronic treatment with levodopa elevated the striatal A_{2A} receptor expression in the lesioned side, as compared to the intact side. More importantly, CSC treatment downregulated the striatal adenosine A_{2A} receptor expression of the lesioned side in the levodopa treated animals, bringing the A_{2A} expression level down to the sham-lesioned and 6-OHDA-lesioned group level. This result was further confirmed by western blot analysis. To keep a stringent internal control, the striatal extracts from the lesioned and unlesioned sides of each individual animal were tested in parallel on the same gel, and the result was expressed as the ratio of the lesioned to the unlesioned side. The abundance of A_{2A} receptor was increased to 204.60±14.55% in the lesioned striatum relative to the unlesioned striatum after the chronic treatment with levodopa, which is significantly higher compared to the sham and 6-OHDA-lesioned rats ($P<0.05$). Co-administration of CSC with levodopa to 6-OHDA-lesioned rats decreased the relative expression ratio of A_{2A} receptor in striatal membrane to 90.40±6.52%, which was significantly lower than those 6-OHDA-lesioned rats treated with levodopa plus vehicle ($P<0.05$), and no statistically significant difference was found between the co-administrated group and the sham-lesioned or the 6-OHDA-lesioned group ($P>0.05$) (Fig. 3).

2.3. *CSC restored DARPP-32 and ERK1/2 phosphorylation level after levodopa treatment*

Since CSC reversed the effect of levodopa on A_{2A} receptor expression, we further studied its effect on potential downstream pathway components. We found there was no significant difference in the abundance of DARPP-32 or ERK1/2 in the striatum of sham, 6-OHDA-lesioned, levodopa plus vehicle-treated, or levodopa plus CSC-treated rats ($P>0.05$). However, analysis of DARPP-32 phosphorylation at Thr75 site and phosphorylation of ERK1/2 revealed some striking alterations (Fig. 4). In the 6-OHDA-lesioned rats, the abundance of DARPP-32 phosphorylation at Thr75 site was increased to

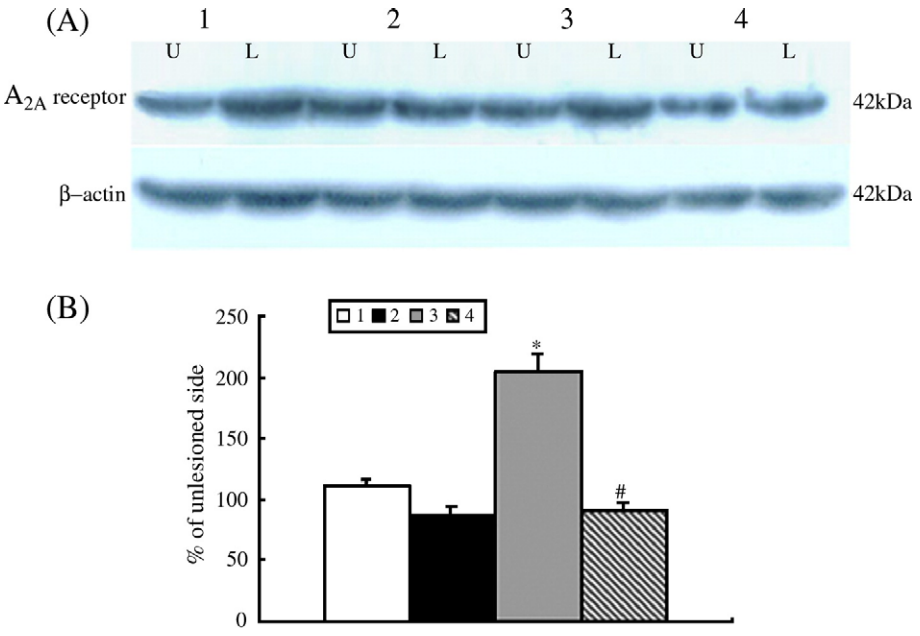

Fig. 3 – The western blotting results confirmed the effect of administration of CSC on the levodopa-induced changes in striatal adenosine A_{2A} receptor expression. The proteins were analyzed from sham (1), 6-OHDA-lesioned (2), 6-OHDA–lesioned plus levodopa treated (3), and 6-OHDA–lesioned plus levodopa in combination with CSC-treated (4) rats. U = unlesioned striatum, L = lesioned striatum. $^{*}P<0.05$ and $^{\#}P<0.05$ compared with 6-OHDA-lesioned, and 6-OHDA-lesioned plus levodopa rats, respectively.

159.90±7.22% in the lesioned striatum relative to the unlesioned striatum, which was significantly higher than the sham group ($P<0.05$). Chronic treatment of lesioned rats with levodopa downregulated phosphorylation of DARPP-32 at Thr75 site to 52.60±4.45% in the lesioned striatum, a level significantly lower than the 6-OHDA-lesioned group ($P<0.05$). CSC plus levodopa treatment of lesioned rats elevated the phosphorylation of DARPP-32 at Thr75 site to 174.20±9.94%, which was significantly higher compared to 6-OHDA-lesioned rats treated with levodopa plus vehicle ($P<0.05$). This level was not significantly different from the 6-OHDA-lesioned rats treated with vehicle ($P>0.05$).

The phosphorylation of ERK1/2 was also altered by the treatments. The 6-OHDA lesion induced a significant downregulation of the phosphorylation of ERK1/2 to 68.28±7.42% compared to the sham-lesioned group ($P<0.05$). The chronic treatment of 6-OHDA-lesioned rats with levodopa significantly increased the phosphorylation of ERK1/2 to 160.37±10.54% compared to the 6-OHDA-lesioned rats treated with vehicle ($P<0.05$). The CSC plus levodopa administration to lesioned rats normalized the phosphorylation of ERK1/2 to 98.36±3.20%, which was significantly lower than the 6-OHDA-lesioned rats treated with levodopa plus vehicle ($P<0.05$), and no significant difference was found between the co-administration group and the sham-lesioned group ($P>0.05$).

3. Discussion

Rats rendered hemi-Parkinsonian by 6-OHDA and subsequently treated twice daily with levodopa developed a progressive shortening in the duration of their locomotor response to a challenge dose of levodopa, mimicking the wearing-off phenomena in patients with Parkinson's disease (Henry et al., 1998; Oh and Chase, 2002). Here we found that, the rats chronically treated with levodopa exhibited a truncated rotational response while administration of CSC with levodopa restored the response duration to levels approximating those before the initiation of the chronic levodopa regimen. Furthermore, we found that there was no tolerance in the effect of CSC, as the reversion was sustained even after the subacute CSC administration. However, we found no effect of the injection of CSC on the abnormal involuntary movement (AIM) scores, which has been used as an index for the severity of the levodopa-induced dyskinesia (data not shown), indicating that the motor fluctuation and AIM may not be originated by the same mechanism (Jenner, 2003). Our results are consistent with a recent study (Bove et al., 2002) showing that CSC could reverse the shortening of the motor duration induced by levodopa administration. In advanced patients, A_{2A} antagonist has been shown to significantly prolong the duration of the "on" phase (Hauser et al., 2003; Kostic et al., 1999).

The mechanism by which CSC reverses the motor fluctuation induced by levodopa is not clear. We analyzed the striatal A_{2A} receptor expression in the four groups to explore the underlying mechanisms. Our result showed that in the 6-OHDA-lesioned group, the expression level of adenosine A_{2A} receptor was not significantly different from sham-lesioned group, but the levodopa treatment increased the striatal A_{2A} expression significantly. The following subacute CSC treatment not only reversed the shortening of motor duration induced by levodopa, but also reversed the upregulation of striatal adenosine A_{2A} receptor expression to the level of the

sham-lesioned and 6-OHDA-lesioned groups. The result is in accordance with the recent report (Tomiyama et al., 2004) that intermittent treatment with levodopa increases adenosine A_{2A} receptor mRNA levels in the dopamine-depleted striatum of 6-OHDA-lesioned rats. Furthermore, it has been shown that adenosine A_{2A} receptor knockout mice are not sensitized by levodopa treatment (Fredduzzi et al., 2002). Taken together, these findings provide strong evidence that overactivation of A_{2A} receptor participates in the induction of levodopa-induced behavioral sensitization.

Adenosine A_{2A} receptor is positively coupled to adenylyl cyclase via the olfactory neuron specific G protein (G_{olf}) (Ferre et al., 2004), and their activation stimulates the cAMP pathway (Kull et al., 2000; Fredholm, 1997). Recent studies have implicated the activation of cAMP/PKA/DARPP-32 pathway in the levodopa-induced motor complications (Santini et al., 2007). Our results showed that the DARPP-32 phosphorylation at site Thr75 was enhanced in dopamine depletion plus vehicle-treated rats. Thr75 is phosphorylated by cyclin-dependent kinase 5 (Cdk5) and dephosphorylated by protein phosphatase 2A (PP2A). PP2A activity may be reduced following dopamine depletion due to the loss of a D1-like receptor activated PKA-dependent activation of PP2A (Nishi et al., 2000), or due to CaMKII-dependent inhibition of PP2A (Fukunaga et al., 2000).Our results also demonstrated that the subsequent levodopa treatment downregulated the Thr75 phosphorylation, which was consistent with previous studies (Brown et al., 2005). The decrease of the DARPP-32 phosphorylation at Thr75 site was more likely caused by the hyperactivating PKA and thus increased phosphorylation of DARPP-32 at Thr34 as a result of supersensitivity of the D1 receptor induced by the prolonged supply of exogenous dopamine, as demonstrated by the recent studies (Aubert et al., 2005; Santini et al., 2007).However, other possible mechanisms to explain the decrease of DARPP-32 phosphorylation at the Thr75 site by chronic administration of levodopa need to be taken into account as well. As we demonstrated that adenosine A_{2A} receptor was upregulated in the lesioned side of 6-OHDA lesion plus levodopa treatment group, it is reported that A_{2A} receptor agonist CGS21680 decreases the DARPP-32 phosphorylation at the Thr75 site in striatal slices (Lindskog et al., 2002). Therefore,

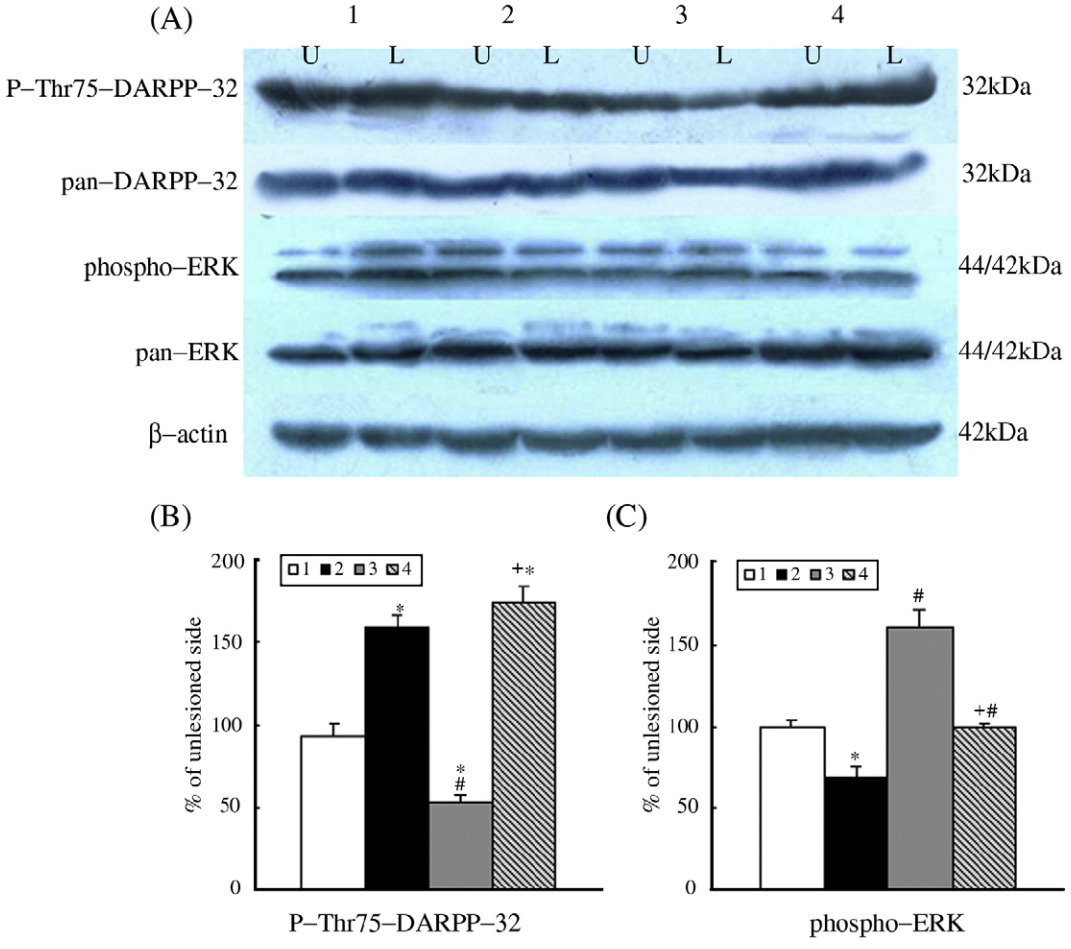

Fig. 4 – Effect of administration of CSC on the levodopa-induced changes in striatal P-Thr75-DARPP-32 and phospho-ERK expression. Panel (A) The proteins were analyzed by western blotting from sham (1), 6-OHDA-lesioned (2), 6-OHDA-lesioned plus levodopa treated (3), and 6-OHDA-lesioned plus levodopa in combination with CSC-treated (4) rats. Panel (B) Bands were scanned and their optical density quantified by densitometry and the value of lesioned side expressed as percent of unlesioned striatum(lesioned/unlesioned × 100% ± SEM). U = unlesioned striatum, L = lesioned striatum. $^{*}P<0.05$, $^{\#}P<0.05$, and $^{+}P<0.05$ compared with sham, 6-OHDA-lesioned, and 6-OHDA-lesioned plus levodopa rats, respectively.

the decrease of striatal Thr75 phosphorylation in the 6-OHDA+levodopa group is possibly related to the upregulation of the adenosine A_{2A} receptor in the lesioned side.

It has been reported that caffeine, a nonselective antagonist for A_{2A} receptors, produced a dose-dependent increase in the state of phosphorylation of DARPP-32 at the Thr75 site in the striata of intact mice, and this effect is primarily dependent on the A_{2A} receptor and is critically involved in the stimulant action of caffeine (Lindskog et al., 2002). To explore the involvement of adenosine A_{2A} receptor in the regulation of DARPP-32 activity in levodopa-induced motor fluctuation, we examined the phosphorylation of DARPP-32 at the Thr75 site in the striatum of the lesioned rats treated with CSC. Our results showed that, following the subacute CSC injection, the phosphorylation of DARPP-32 at Thr75 was enhanced in the striatum of CSC-treated rats compared to the levodopa plus vehicle-treated group. These results confirmed that the subacute administration of CSC, which could block the adenosine A_{2A} receptor function, could also modulate the phosphorylation of DARPP-32 at Thr75 in the 6-OHDA-lesioned rats treated with levodopa. Combined with the fact that the A_{2A} receptor is selectively involved in the indirect pathway, we propose that CSC would restore the duration of contralateral rotational behavior by blocking adenosine A_{2A} receptors and reducing tonic activation of the cAMP/PKA pathway in striato-Gpe neurons.

Increasing evidence has suggested the ERK signaling pathway is involved in the long-term levodopa therapy-induced motor complications. Our results showed that the dopamine depletion downregulated the phosphorylation of ERK in the striatum, while the subsequent levodopa treatment induced an increase in ERK phosphorylation in the rats with motor alteration. This result is in accordance with a recent report (Westin et al., 2007), which shows an increase of ERK phosphorylation in LID rats, and the phospho-ERK level is also related to the AIM scores. It has been reported that the selective A_{2A} receptor agonist, CGS 21680, causes an upregulation of phospho-ERK1/2 in striatal slices, while the injection of A_{2A} antagonist caffeine does not significantly reduce the phospho-Thr202-ERK1 and phospho-Thr183-ERK2 levels *in vivo* (Sahin et al., 2007). Our result is apparently paradoxical in that the selective adenosine A_{2A} receptor antagonist CSC, while reversed the shortening of duration, attenuated the upregulation of phospho-ERK1/2 induced by chronic levodopa treatment. Nevertheless, it is consistent with a recent study demonstrating that blockade of adenosine A_{2A} receptors with caffeine or a selective A_{2A} antagonist counteracts the striatal activation of MAPK (ERK1/2 phosphorylation) induced by the *in vivo* stimulation of corticostriatal afferents (Quiroz et al., 2006). In addition, levodopa-induced motor complication is indeed associated with an excessive activation of motor and premotor cortical areas (Rascol et al., 1998). Since it is reported that the phosphorylation of ERK is partially dependent on the activation of the PKA/DARPP-32 pathway, the modification of ERK phosphorylation by CSC could be related to its effect on blocking adenosine A_{2A} receptor and reducing tonic activation of the cAMP/PKA pathway in striato-Gpe neurons.

Overall, our results demonstrated that CSC, while reversed the motor alteration induced by chronic levodopa treatment, attenuated the upregulation of adenosine A_{2A} receptor expression in the lesioned side of PD rats after levodopa treatment. CSC also attenuated the overactivation of the PKA and ERK pathways as evident by the downregulation of ERK phosphorylation and upregulation of DARPP-32 Thr75 phosphorylation. It is believed that the phosphorylation of DARPP-32 at Thr75 site and ERK1/2 is related to the overactivity of direct pathway (Westin et al., 2007). Our results suggested that modulation of the indirect pathway by the adenosine A_{2A} receptor could also influence the phosphorylation of the DARPP-32 and ERK1/2. Given the fact that phosphorylation of DARPP-32 is critically involved in the stimulant action of caffeine (Lindskog et al., 2002), we confirmed that DARPP-32 and ERK were involved in the levodopa-induced motor complications not only at the level of the direct, striato-nigral/Gpi pathway (which depends on dopamine D_1 receptors), but also at the level of the indirect, striato-Gpe pathway (which depends on adenosine A_{2A} receptors). The effect of CSC to reverse the shortening of the duration of contralateral rotation may be related to the modulation of the PKA and ERK pathways.

4. Experimental procedures

4.1. *Animals*

Fifty adult rats (Sprague–Dawley females, 200–250 g) were used in the present study. Rats were housed with free access to water and standard rat chow at the Institute of Experimental Animal Science, Shanghai Jiaotong University School of Medicine. All protocols involving animals were approved by the Institutional Review Board of Xinhua Hospital and were performed according to the guidelines of the National Institutes of Health for the care and use of laboratory animals (NIH publication No. 80–23). All efforts were made to minimize the number of animals used and their suffering.

4.2. *Reagents*

6-OHDA, apomorphine, ascorbic acid, levodopa methyl ester, benserazide, 8-(3-Chlorostyryl) caffeine (CSC) were from Sigma Chemical Co. (St. Louis, MO). Antibodies against DARPP-32, phosho-Thr75-DARPP-32, pan-ERK1/2 and phospho-ERK1/2 were obtained from Cell Signaling. Rabbit polyclonal antibody against adenosine A_{2A} receptor were obtained from Santa Cruz Biotechnology Inc. Horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody was ordered from Vector Laboratories (Burlingame, CA). Rabbit anti-β-actin antibody was from Abcam Inc. (Cambridge, UK). Cell lysis buffer was from Beyotime Biotechnology, China.

4.3. *6-OHDA lesion surgery and rotational screening*

To create the 6-OHDA lesion, the rat was anesthetized with pentobarbital (50 mg/kg body weight, i.p.) and its head was fixed in a stereotactic apparatus (Narishige, Japan) with the incisor bar. Nigrostriatal lesions were produced by injecting 8 μg of 6-OHDA dissolved in 4 μl of 0.9% physiological saline containing 0.02% ascorbic acid into the right medial forebrain bundle with a 10-μl Hamilton syringe. The coordinates used

were: bregma 4.5 mm; lateral 0.9 mm; dura 7.5 mm. These numbers were in accordance with a rat brain atlas (Paxinos and Watson, 1998). The injection was administered within a period of 5 min and the needle of the Hamilton syringe was kept in position for a further 10 min following the deposit in order to prevent back filling along the injection tract. As controls, 10 rats underwent a sham lesion procedure in which only the vehicle for 6-OHDA was injected into the medial forebrain bundle. Rat rotation tests were performed in order to access the successfulness of the 6-OHDA lesion 3 weeks after surgery. The lesioned rats were injected with apomorphine (0.25 mg/kg in physiological saline, i.p.) and were placed in a stainless steel bowl. The numbers of rotations were scored visually for each rat. Those rats exhibiting a vigorous rotational response (>100 total turns) to apomorphine were regarded as successfully lesioned animals and selected for further study. It has been previously demonstrated that rats meeting this criterion have a greater than 95% depletion of striatal dopamine.

4.4. *Protocol of treatment*

Four groups of rats were included in our study. Sham-lesioned rats treated with saline for 29 days served as sham-lesioned control group ($n=10$). 6-OHDA-lesioned rats treated with saline for 29 days served as the 6-OHDA-lesioned control group ($n=10$). The rest 6-OHDA-lesioned rats were treated with levodopa methyl ester (25 mg/kg with 6.25 mg/kg benserazide, dissolved in saline, twice daily, i.p.) for 22 days. On day 23, the animals were randomly assigned into levodopa plus CSC-treated group ($n=10$) and levodopa plus vehicle-treated group ($n=10$) to receive the following treatments accordingly for one additional week: (i) levodopa plus the adenosine A_{2A} antagonist CSC (2.5 mg/kg, twice daily, i.p., $n=10$) (ii) levodopa plus vehicle ($n=10$). Rotational behavior was measured immediately following the administration of levodopa. The duration of the rotational response was measured as the time between the first 5-min interval when turning exceeded 20% of the peak rate and the first interval when turning fell below 20% of the peak rate. The peak intensity of rotation was measured as the peak number of contralateral turns in any 5-min interval. Rotational behavior was measured on days 1, 22, 23, and 29 of levodopa treatment.

4.5. *Immunochemistry*

12 h after the last drug treatment, three to five rats from each group were deeply anaesthetized with pentobarbital (50 mg/kg body weight, i.p.), then sacrificed by transcardial perfusion with physiological saline, followed by phosphate-buffered 4% paraformaldehyde (pH7.4) for immunohistochemical studies. Brains were rapidly extracted, immersed in the above fixative overnight and dehydrated by graded alcohol. Then, brains were embedded in paraffin, cut into 5-μm-thick coronal sections and subjected to immunohistochemistry assays using the avidin–biotin–peroxidase complex technique (Vector Laboratories) with diaminobenzidine as a substrate. The sections were incubated with an affinity-purified polyclonal antibody against adenosine A_{2A} receptor overnight at 4 °C. The staining specificity was assessed by omission of the primary antibody.

For quantitative analysis of immunostaining intensity of adenosine A_{2A} receptor, we computed the integrated optical density (IOD). Images from 6 random, non-overlapping areas from each section were captured with an Olympus BH-2 microscope fitted with a MicroImage video camera at ×400 magnifications to gain a mean value for each section. Digitally fixed images were analyzed using an image analyzer (Image Pro Plus).

4.6. *Western blot*

The rest animals were deeply anaesthetized with pentobarbital (50 mg/kg body weight, i.p.) 30 min after the last drug administration. The brains were rapidly extracted, and the left and right striatum were dissected and frozen at −80 °C for western blots. Sham-lesioned and 6-OHDA-lesioned animals that received chronic vehicle but not chronic levodopa treatment served as controls. Frozen striatal tissue was lysed in 1.5-ml microtube with lysis buffer [20 mM Tris–HCl, PH7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na_3VO_4, 0.5 μg/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride (PMSF)]. The tissue was homogenized by sonication. The supernatant was collected after a centrifugation for 10 min at 10,000 *g*. Protein concentrations was measured with a BCA assay kit (Rockford, IL). A 15 μg protein sample was mixed with 2× sample buffer (4% SDS, 20% glycerol, 10% mercaptoethanol and a trace amount of Bromphenol Blue dye in 125 mM Tris–HCl, pH 6.8) and heated to 100 °C for 5 min. The samples were then loaded onto an SDS polyacrylamide electrophoresis gel (15%). After electrophoresis the proteins was electrotransferred onto a 0.2-mm PVDF membrane (Bio-Rad, Hercules, CA) in a transfer buffer (25 mM Tris–HCl, 192 mM glycine and 20% methanol) with 250 mA current for 90 min at 4 °C. Residual binding sites on the membrane were blocked by incubation in 1× TBS with 0.1% Tween 20 and 5% skimmed milk. The membranes were then incubated with the primary antibodies anti-adenosine A_{2A} receptor (1:800), anti-DARPP-32 (1:800), anti-ERK (1:800), anti-phospho-Thr75-DARPP-32 (1:500), anti-phospho-ERK (Thr202/Tyr204) (1:500), or anti-β-actin (1:1000) at 4 °C overnight. Omission of the primary antibodies served as negative controls. The primary antibody was then removed and the membrane were washed three times in 1× TBS with 0.1% Tween 20. To detect the antibody reactions, the membrane was incubated for 2 h with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody diluted 1:1000 in 1× TBS with 0.1% Tween 20 and 5% skim milk at room temperature. The membrane was then washed extensively in 1× TBS with 0.1% Tween 20, and was then placed in the chemiluminescent working solution (ECL detection reagents) for 5 min at room temperature. The membrane was then removed from the working solution, and exposed to Kodak Biomax MS-1 films for 0.5–3 min.

4.7. *Statistics*

The experimental data were presented as mean ± SEM. Paired Student's *t*-tests was used to analyze the immunohistochemical data within groups. Analysis of variance (ANOVA) followed by LSD's post hoc comparison tests was employed

to analyze the data among different groups; a value of $P<0.05$ was considered significant.

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REFERENCES

Arslan, G., Fredholm, B.B., 2000. Stimulatory and inhibitory effects of adenosine A(2A) receptors on nerve growth factor-induced phosphorylation of extracellular regulated kinases 1/2 in PC12 cells. Neurosci. Lett. 292, 183–186.

Aubert, I., Guiqoni, C., Håkansson, K., Li, Q., Dovero, S., Barthe, N., Bioulac, B.H., Gross, C.E., Fisone, G., Bloch, B., Bezard, E., 2005. Increased D1 dopamine receptor signaling in levodopa-induced dyskinesia. Ann. Neurol. 57, 17–26.

Berhow, M.T., Hiroi, N., Nestler, E.J., 1996. Regulation of ERK (extracellular signal regulated kinase), part of the neurotrophin signal transduction cascade, in the rat mesolimbic dopamine system by chronic exposure to morphine or cocaine. J. Neurosci. 16, 4707–4715.

Bibb, J.A., Snyder, G.L., Nishi, A., Yan, Z., Meijer, L., Fienberg, A.A., Tsai, L.H., Kwon, Y.T., Girault, J.A., Czernik, A.J., Huganir, R.L., Hemmings Jr, H.C., Nairn, A.C., Greengard, P., 1999. Phosphorylation of DARPP-32 by Cdk5 modulates dopamine signalling in neurons. Nature 402, 669–671.

Bove, J., Marin, C., Bonastre, M., Tolosa, E., 2002. Adenosine A2A antagonism reverses levodopa-induced motor alterations in hemiparkinsonian rats. Synapse 46, 251–257.

Brown, A.M., Deutch, A.Y., Colbran, R.J., 2005. Dopamine depletion alters phosphorylation of striatal proteins in a model of Parkinsonism. Eur. J. Neurosci. 22, 247–256.

Deak, M., Clifton, A.D., Lucocq, L.M., Alessi, D.R., 1998. Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. Embo J. 17, 4426–4441.

Dunn, K.L., Espino, P.S., Drobic, B., He, S., Davie, J.R., 2005. The Ras-MAPK signal transduction pathway, cancer and chromatin remodeling. Biochem. Cell. Biol. 83, 1–14.

Fredduzzi, S., Moratalla, R., Monopoli, A., Cuellar, B., Xu, K., Onqini, E., Impaqnatiello, F., Schwarzschild, M.A., Chen, J.F., 2002. Persistent behavioral sensitization to chronic requires A_{2A} adenosine receptors. J. Neurosci. 22, 1054–1062.

Ferre, S., Ciruela, F., Canals, M., Marcellino, D., Burgueno, J., Casadó, V., Hillion, J., Torvinen, M., Fanelli, F., Benedetti, P.d.P., Goldberg, S.R., Bouvier, M., Fuxe, K., Agnati, L.F., Lluis, C., Franco, R., Woods, A., 2004. Adenosine A2A-dopamine D2 receptor-receptor heteromers. Targets for neuro-psychiatric disorders. Parkinsonism Relat. Disord. 10, 265–271.

Fredholm, B.B., 1997. Activation of adenylate cyclase from rat striatum and tuberculum olfactorium by adenosine. Med. Biol. 55, 262–267.

Fukunaga, K., Muller, D., Ohmitsu, M., Bakó, E., DePaoli-Roach, A.A., Miyamoto, E., 2000. Decreased protein phosphatase 2A activity in hippocampal long-term potentiation. J. Neurochem. 74, 807–817.

Grondin, R., Bedard, P.J., Hadj Tahar, A., Grégoire, 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.

Greengard, P., Allen, P.B., Nairn, A.C., 1999. Beyond the dopamine receptor: the DARPP-32/protein phosphatase-1 cascade. Neuron 23, 435–447.

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

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

Jenner, P., 2003. The contribution of the MPTP-treated primate model to the development of new treatment strategies for Parkinson's disease. Parkinsonism Relat. Disord. 9, 131–137.

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 or with selective D1 or D2 dopamine agonists increases antiparkinsonian activity but not dyskinesia in MPTP-treated monkeys. Exp. Neurol. 162, 321–327.

Keyse, S.M., 2000. Protein phosphatases and the regulation of mitogen-activated protein kinase signalling. Curr. Opin. Cell Biol. 12, 186–192.

Kostic, S., Svetel, M., Sternic, N., Dragasevic, N., Przedborski, S., 1999. Theophylline increases "on" time in advanced parkinsonian patients. Neurology 52, 1916.

Kull, B., Svenningsson, P., Fredholm, B.B., 2000. Adenosine A(2A) receptors are colocalized with and activate G(olf) in rat striatum. Mol. Pharmacol. 58, 771–777.

Lang, A.E., Obeso, J.A., 2004. Challenges in Parkinson's disease: restoration of the nigrostriatal dopamine system is not enough Lancet Neurology 3, 309–316.

Lindskog, M., Svenningsson, P., Pozzi, L., Kim, Y., Fienberg, A.A., Bibb, J.A., Fredholm, B.B., Nairn, A.C., Greengard, P., Fisone, G., 2002. Involvement of DARPP-32 phosphorylation in the stimulant action of caffeine. Nature 418, 774–776.

Marshall, C.J., 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80, 179–185.

Nishi, A., Bibb, J.A., Snyder, G.L., Higashi, H., Nairn, A.C., Greengard, P., 2000. Amplification of dopaminergic signaling by a positive feedback loop. Proc. Natl. Acad. Sci. U. S. A. 97, 12840–12845.

Ouimet, C.C., Langley-Gullion, K.C., Greengard, P., 1998. Quantitative immunocytochemistry of DARPP-32-expressing neurons in the rat caudatoputamen. Brain Res. 808, 8–12.

Oh, J.D., Chase, T.N., 2002. Glutamate-mediated striatal dysregulation and the pathogenesis of motor response complications in Parkinson's disease. Amino Acids 23, 133–139.

Paxinos, G., Watson, C., 1998. The Rat Brain in Stereotaxic Coordinates, 4th ed. Academic Press.

Pavon, N., Martin, A.B., Mendialdua, A., Moratalla, R., 2006. ERK phosphorylation and FosB expression are associated with L-DOPA-induced dyskinesia in hemiparkinsonian mice. Biol. Psychiatry 59, 64–74.

Quiroz, C., Gomes, C., Pak, A.C., Ribeiro, J.A., Goldberg, S.R., Hope, B.T., Ferre, S., 2006. Blockade of adenosine A2A receptors prevents protein phosphorylation in the striatum induced by cortical stimulation. J. Neurosci. 26, 10808–10812.

Rascol, O., Sabatini, U., Brefel, C., Fabre, N., Rai, S., Senard, J.M., Celsis, P., Viallard, G., Montastruc, J.L., Chollet, F., 1998. Corticalmotor overactivation in parkinsonian patients with L-dopa-induced peak-dose dyskinesia. Brain 121, 527–533.

Schiffmann, S.N., Vanderhaeghen, J.J., 1993. Adenosine A_{2A} receptors regulate the gene expression of striatopallidal and striatonigral neurons. J. Neurosci. 13, 1080–1087.

Svenningsson, P., Le Moine, C., Kull, B., Sunahara, R., Bloch, B., Fredholm, B.B., 1997. Cellular expression of adenosine A_{2A} receptor messenger RNA in the rat central nervous system with special reference to dopamine innervated areas. Neuroscience 80, 1171–1185.

Santini, E., Valjent, E., Usiello, A., Carta, M., Borgkvist, A., Girault, J.A., Hervé, D., Greengard, P., Fisone, G., 2007. Critical involvement of cAMP/DARPP-32 and extracellular signal-regulated protein kinase signaling in L-DOPA-induced dyskinesia. J. Neurosci. 27, 6995–7005.

Sahin, B., Galdi, S., Hendrick, J., Greene, R.W., Snyder, G.L., Bibb, J.A., 2007. Evaluation of neuronal phosphoproteins as effectors of caffeine and mediators of striatal adenosine A_{2A} receptor signaling. Brain Res. 1129, 1–14.

Tomiyama, M., Kimura, T., Maeda, T., Tanaka, H., Kannari, K., Baba, M., 2004. Upregulation of striatal adenosine A2A receptor mRNA in 6-hydroxydopamine-lesioned rats intermittently treated with L-DOPA. Synapse 2, 218–222.

Valjent, E., Corvol, J.C., Pages, C., Besson, M.J., Maldonado, R., Caboche, J., 2000. Involvement of the extracellular signal-regulated kinase cascade for cocaine-rewarding properties. J. Neurosci. 20, 8701–8709.

Valjent, E., Pages, C., Herve, D., Girault, J.A., Caboche, J., 2004. Addictive and non-addictive drugs induce distinct and specific patterns of ERK activation in mouse brain. Eur. J. Neurosci. 19, 1826–1836.

Valjent, E., Pascoli, V., Svenningsson, P., Paul, S., Enslen, H., Corvol, J.C., Stipanovich, A., Caboche, J., Lombroso, P.J., Nairn, A.C., Greengard, P., Hervé, D., Girault, J.A., 2005. Regulation of a protein phosphatase cascade allows convergent dopamine and glutamate signals to activate ERK in the striatum. Proc. Natl. Acad. Sci. U. S. A. 102, 491–496.

Wyatt, A.W., Steinert, J.R., Wheeler-Jones, C.P., Morgan, A.J., Sugden, D., Pearson, J.D., Sobrevia, L., Mann, G.E., 2002. Early activation of the p42/p44MAPK pathway mediates adenosine-induced nitric oxide production in human endothelial cells: a novel calcium-insensitive mechanism. Faseb J. 16, 1584–1594.

Westin, J.E., Vercammen, L., Strome, E.M., Konradi, C., Cenci, M.A., 2007. Spatiotemporal pattern of striatal ERK1/2 phosphorylation in a rat model of L-DOPA-induced dyskinesia and the role of dopamine D1 receptors. Biol. Psychiatry 62, 800–810.