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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8-(3-chlorostryryl) caffeine (CSC), a selective adenosine A2A 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 A2A 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 A2A 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 A2A Receptor and downstream DARPP-32 and ERK1/2 signaling pathway.

Keywords: 8-(3-chlorostryryl) caffeine (CSC) Parkinson’s disease Motor complications Phosphorylation Adenosine A2A receptor

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).
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 antagonism 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 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...
the brains of drug-treated and control animals. Immunohis-
tochemistry tests revealed that, the adenosine A2A receptor
was predominantly located in the cell membrane (Fig. 2). 6-
OHDA lesion did not alter the level of striatal A2A 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 A2A receptor expression in
the lesioned side, as compared to the intact side. More
importantly, CSC treatment downregulated the striatal ade-
nosine A2A receptor expression of the lesioned side in the
levodopa treated animals, bringing the A2A 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 A2A 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 levo-
dopa to 6-OHDA-lesioned rats decreased the relative expres-
sion ratio of A2A 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-
administered 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 A2A receptor
expression, we further studied its effect on potential down-
stream 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 altera-
tions (Fig. 4). In the 6-OHDA-lesioned rats, the abundance of
DARPP-32 phosphorylation at Thr75 site was increased to
159.90±7.22% in the lesioned striatum relative to the unle-
sioned 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 down-
regulation 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 signifi-
cantly increased the phosphorylation of ERK1/2 to 160.37±
10.54% compared to the 6-OHDA-lesioned rats treated with
vehicle (P<0.05). This level was not significantly different from the 6-OHDA-lesioned rats
with vehicle (P>0.05).

The mechanism by which CSC reverses the motor fluctua-
tion induced by levodopa is not clear. We analyzed the striatal
A2A 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 A2A
receptor was not significantly different from sham-lesioned
A2A receptor expression to the level of the
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, A2A antagonist has been shown to
significantly prolong the duration of the “on” phase (Hauser
et al., 2003; Kostic et al., 1999).

3. Discussion

Rats rendered hemi-Parkinsonian by 6-OHDA and subse-
quently 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
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OHDA-lesioned group, the expression level of adenosine A2A
receptor was not significantly different from sham-lesioned
A2A 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 \(A_{2\alpha}\) receptor mRNA levels in the dopamine-depleted striatum of 6-OHDA-lesioned rats. Furthermore, it has been shown that adenosine \(A_{2\alpha}\) receptor knockout mice are not sensitized by levodopa treatment (Fredduzzi et al., 2002). Taken together, these findings provide strong evidence that overactivation of \(A_{2\alpha}\) receptor participates in the induction of levodopa-induced behavioral sensitization.

Adenosine \(A_{2\alpha}\) 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 \(A_{2\alpha}\) receptor was upregulated in the lesioned side of 6-OHDA lesion plus levodopa treatment group, it is reported that \(A_{2\alpha}\) receptor agonist CGS21680 decreases the DARPP-32 phosphorylation at the Thr75 site in striatal slices (Lindskog et al., 2002). Therefore,

![Figure 4](image)

**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 \(\times 100\% \pm SEM\)). 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 A2A receptor in the lesioned side.

It has been reported that caffeine, a nonselective antagonist for A2A 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 A2A receptor and is critically involved in the stimulant action of caffeine (Lindskog et al., 2002). To explore the involvement of adenosine A2A 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 A2A 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 A2A receptor is selectively involved in the indirect pathway, we propose that CSC would restore the duration of contralateral rotational behavior by blocking adenosine A2A 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 A2A receptor agonist, CGS 21680, causes an upregulation of phospho-ERK1/2 in striatal slices, while the injection of A2A 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 A2A 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 A2A receptors with caffeine or a selective A2A antagonist counteracts the striatal activation of MAPK (ERK1/2 phosphorylation) induced by the in vivo stimulation of corticostriatal afferents (Quirao et al., 2006). In addition, levodopa-induced motor complication is indeed associated with an excessive activation of motor and premotor cortical areas (Rasci 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 A2A 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 A2A 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 A2A 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, striatonigral/Gpi pathway (which depends on dopamine D1 receptors), but also at the level of the indirect, striato-Gpe pathway (which depends on adenosine A2A 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, benserase, 8-(3-Chlorostyryl) caffeine (CSC) were from Sigma Chemical Co. (St. Louis, MO). Antibodies against DARPP-32, phospho-Thr75-DARPP-32, pan-ERK1/2 and phospho-ERK1/2 were obtained from Cell Signaling. Rabbit polyclonal antibody against adenosine A2A 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 for the 6-OHDA lesion were: 4.3. 6-OHDA lesion surgery and rotational screening

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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 success 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 A2A 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 time between 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. Immunohistochemistry

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 paraformaldehyde (pH 7.4) for immunohistochemical studies. Brains were rapidly extracted, immersed in the above fixative over-night 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 A2A receptor overnight at 4 °C. The staining specificity was assessed by omission of the primary antibody.

For quantitative analysis of immunostaining intensity of adenosine A2A 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, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na3VO4, 0.5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl 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 A2A 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 was 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% skimmed 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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