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GINKGO BILOBA EXTRACT (EGb 761) MODULATES THE EXPRESSION OF DOPAMINE-RELATED GENES IN 1-METHYL-4-PHENYL-1,2,3,6-TETRA-HYDROPYRIDINE-INDUCED PARKINSONISM IN MICE

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Abstract—1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes nigrostriatal dopaminergic neurotoxicity and behavioral impairment in rodents similar to Parkinson's disease. The MPTP mouse model is widely used to evaluate new protective agents. EGb 761 is a well-defined mixture of active compounds extracted from *Ginkgo biloba* leaves according to a standardized procedure. We have shown that EGb 761 attenuates the loss of striatal dopamine levels and prevents the neurodegeneration of the nigrostriatal pathway induced by MPTP. This finding shows that neuroprotective effects of EGb 761 act, in part, on the dopamine system. Therefore, this study investigates whether EGb 761 exerts dopaminergic neuroprotection through the regulation of dopamine-related gene expression in MPTP-induced Parkinsonism. Male C57BL/6J mice were injected with MPTP (30 mg/kg, i.p.) for 5 days and later with EGb 761 (40 mg/kg, i.p.) daily for 18 days. The expression of selected genes was evaluated in the striatum and midbrain by quantitative PCR. The genes for tyrosine hydroxylase (*Th*), vesicular monoamine transporter 2 (*Vmat2*), dopamine transporter (*Dat*), dopamine D2 receptor (*Da-d2r*), and transcription factors (*Pitx3 and Nurr1*) related to dopamine neurotransmission were selected for the analysis. EGb 761 administration to MPTP-treated mice protected *Th* (41%), *Vmat2* (15%), *Dat* (102%), *Da-d2r* (46%), *Pitx3* (63%), and *Nurr1* (148%) mRNA levels in the midbrain, all of which were up-regulated. However, EGb 761 partially reversed the MPTP effect exclusively for *Th* (48%) and *Nurr1* (96%) mRNA in the striatum. Only *Th* and *Nurr1* mRNA and protein levels were regulated by EGb 761 in both regions of the nigrostriatal pathway. This result could be related to the regulation of their transcription. Our results suggest that EGb 761-associated neuroprotection against MPTP neurotoxicity is related to the regulation of the dopamine genes. Moreover, this neuroprotection also involves the regulation of transcription factors such as Nurr1 that are important for the functional maintenance of dopaminergic neurons. **© 2012 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: EGb 761, Parkinson's disease model, MPTP, *Nurr1*, gene expression.

INTRODUCTION

Parkinson's disease (PD) is a neurological disorder characterized by the degeneration and death of the dopaminergic neurons of the nigrostriatal pathway (Jenner, 1989). The death of these neurons results in a decrease in striatal dopamine (DA) content. The cause of neuronal loss is unclear, but oxidative stress via free radical production plays an important role in this process (Owen et al., 1996).

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exposure produces Parkinsonism in humans (Langston et al., 1983). MPTP is a dopaminergic toxin that causes a marked depletion of DA, nigral cell loss and clinical symptoms similar to those of PD (Przedborski et al., 2000). In MPTP-intoxicated humans and nonhuman primates, the beneficial response to levodopa is similar to that observed for PD patients, which is one of the primary reasons for using this toxin as an animal model. Although nonhuman primates MPTP model remains the best due to its similarities to PD patients, most studies have been performed in mouse models, which exhibit striatal DA depletion and a loss of dopaminergic neurons (Przedborski et al., 2000; Blesa et al., 2012). Therefore, the mouse model is the most widely used to evaluate new neuroprotective agents (Anderson et al., 2006). There are different MPTP administration protocols that produce different types of cell death and respond differently to putative neuroprotective agents (Anderson et al., 2006; Antony et al., 2011). Sub-acute MPTP administration is associated primarily with apoptotic cell death (Tatton and Kish, 1997). This effect is likely the result of an impairment in complex I activity leading to oxidative stress and free radical production (Singer et al., 1987; Speciale, 2002). MPTP neurotoxicity is linked, in part, to

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Abbreviations: DA, dopamine; *Dat*, dopamine transporter; *Da-d2r*, dopamine D2 receptor; EGb 761, *Ginkgo biloba* extract; MPTP, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine; *Nurr1*, Nuclear receptor related factor 1; PD, Parkinson's disease; *Pitx3*, Paired-like homeodomain 3; PMSF, phenylmethylsulfonyl fluoride; qPCR, quantitative real-time PCR; TBP, TATA box-binding protein; *Th*, tyrosine hydroxylase; *Vmat2*, vesicular monoamine transporter 2.

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oxidative stress via free radical production (Sriram et al., 1997; Wong et al., 1999). Several antioxidants (radical scavengers) have been reported to protect against MPTP-induced neurotoxicity (Ferger et al., 1998; Mohanakumar et al., 2000; Park et al., 2004). Therefore, agents that are able to inhibit the oxidative stress produced by MPTP may have neuroprotective effects.

The *Ginkgo biloba* extract EGb 761 is the most widely used medicinal plant product in Europe. EGb 761 is used clinically to treat dementia and vasoocclusive and cochleovestibular disorders (von Boetticher, 2011; Herrschaft et al., 2012). A potent antioxidant (Droy-Lefaix et al., 1995; Guidetti et al., 2001) and free radical scavenger (Maitra et al., 1995; Ni et al., 1996), it is a well-known plant extract obtained from the leaves of the *G. biloba* tree according to a standardized procedure (Drieu, 1986). EGb 761 contains flavonoids (24%), terpenoids (6%; ginkgolides A, B, C, M, J, and bilobalide), 5–10% organic acids, and >0.5% proanthocyanidins (Drieu, 1986). The relatively low molecular weights of these compounds potentially permit their penetration of the blood–brain barrier, resulting in a broad spectrum of pharmacological actions in the central nervous system (DeFeudis and Drieu, 2000).

Several studies have demonstrated that EGb 761 prevents the alteration of the neuronal DA system (Yoshitake et al., 2010; Yeh et al., 2011). This extract can also provide effective protection against the damage to midbrain DA neurons arising from MPTP neurotoxicity (Wu and Zhu, 1999; Rojas et al., 2008). We have shown that EGb 761 attenuates the loss of striatal DA levels and prevents the neurodegeneration of the nigrostriatal pathway that is induced by MPTP (Rojas et al., 2008). In that study the neuroprotective effects of EGb 761 against MPTP neurotoxicity were associated with reduced oxidative stress. We also reported that EGb 761 regulates monoamine oxidase (MAO) activity and enhances tyrosine hydroxylase content in MPTP neurotoxicity (Rojas et al., 2004). Moreover, EGb 761 improved the MPTP-induced impairment of locomotion (Rojas et al., 2008), showing that the EGb 761 neuroprotective effect is, in part, acting on the DA system. These studies suggest it as a therapeutic alternative in the future treatment of PD (Rojas et al., 2012). However, there have been few studies regarding the neuroprotective effects of EGb 761 on the dopaminergic neurodegeneration induced by MPTP.

In addition, the nuclear receptor related factor 1 (*Nurr1*) gene, which encodes a transcription factor, is essential for the development and functional maintenance of differentiated dopaminergic neurons (Chung et al., 2002). Nurr1 appears to regulate the expression of the tyrosine hydroxylase (*Th*), dopamine transporter (*Dat*), and vesicular monoamine transporter 2 (*Vmat2*) genes (Jankovic et al., 2005); all of these are important in the synthesis and storage of DA. Nurr1 interacts with another transcription factor, Paired-like homeodomain 3 (Pitx3), that is important in dopaminergic neurons (Martinat et al., 2006). Furthermore, MPTP decreases the gene expression of *Th*, *Dat*, *Vmat2* (Xu et al., 2005), and *Nurr1* (Gibrat et al., 2009).

Therefore, we examined whether EGb 761 exerts dopaminergic neuroprotection through the regulation of DA-related gene expression in an animal model of PD. The analysis of gene expression included *Th*, *Vmat2*, *Dat*, *Da-d2r*, *Pitx3*, *and Nurr1*.

EXPERIMENTAL PROCEDURES

Animals

Experiments were conducted on male C57BL/6J mice (Harlan, Mexico) that were 11–13 weeks of age. The animals were housed 5 per cage and were maintained in standard conditions (12:12 h light/dark cycle, 21 ± 2 °C, relative humidity 40%). They were allowed access to food and water *ad libitum* up until the time of the experiment. All experiments were carried out in accordance with the National Institutes of Health Guide (USA) for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1978). All experiments conformed to regulations specified by the Animal Care and Use Committee of our institution and the standards of the National Institutes of Health of Mexico (NOM-062-ZOO-1999). All efforts were made to reduce the number of animals used and to treat them humanely to minimize their pain and discomfort.

Drugs and treatment

We used a sub-acute MPTP mouse model. Mice received daily intraperitoneal (i.p.) injections of vehicle (saline) or MPTP (30 mg free base/kg body weight; Sigma–Aldrich, St. Louis MO, USA) dissolved in physiological saline for 5 consecutive days to induce Parkinsonism. EGb 761 treatment was performed daily over the course of 18 days starting 24 h after the last MPTP administration. EGb 761 was kindly provided by Schwabe Pharmaceuticals (Karlsruhe, Germany). This extract is well characterized (DeFeudis, 1998) and is used in ongoing clinical trials (DeKosky et al., 2006). EGb 761 was dissolved in physiological saline and the pH adjusted to 7.4. We have reported that EGb 761 (40 mg/kg) produces the highest neuroprotection against the dopaminergic damage produced by MPTP neurotoxicity (Rojas et al., 2008).

The effects of EGb 761 on the expression of DA-related genes in MPTP-induced Parkinsonism were studied in the following 4 experimental groups ($n = 6–8$ mice per group): Group I: saline solution (i.p.) + saline solution (i.p.); Group II: saline solution (i.p.) + EGb 761 (i.p.); Group III: MPTP (i.p.) + saline solution (i.p.); and Group IV: MPTP (i.p.) + EGb 761 (i.p.). Animals in groups I and III received normal saline solution (i.p.), and animals in groups II and IV received EGb 761 (40 mg/kg, i.p.) daily for 18 days. Mice from groups I and II were injected with saline solution (i.p.) and served as controls.

Dissection of tissue and the extraction of total RNA

Mice were sacrificed by cervical dislocation 24 h after the last EGb 761 or saline administration. Brains were removed immediately, and the striatum and midbrain were dissected on ice and immediately transferred to dry ice to preserve RNA integrity. These regions were used to analyze the dopaminergic nigrostriatal pathway affected by MPTP-induced neurodegeneration. Total RNA was isolated from the striatum and midbrain using 800 µl TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions. RNA integrity was determined by agarose gel electrophoresis and the concentration and purity were measured spectrophotometrically (Kingston et al., 1996). Total RNA was converted to single stranded cDNA using 2 µg of

total RNA as a template. Oligo $(dT)_{12-18}$ primer (Invitrogen Life Technologies) and Moloney-murine leukemia virus reverse transcriptase (M-MLV RT; Invitrogen Life Technologies) were used following the manufacturer's instructions.

Quantitative real-time PCR

The quantitative real-time PCR (qPCR) assays of different genes in the striatum and midbrain were analyzed using a Rotor-Gene 6000 instrument (Corbett Research Pty Ltd., Sydney, Australia); the expression levels of *Th, Vmat2*, *Dat*, *Da-d2r*, *Pitx3*, and *Nurr1* were analyzed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference gene to normalize target gene transcript levels. Quantitative PCR reactions were performed using predesigned primer and probes from Applied Biosystems (Foster City, CA, USA). Each assay consisted of TaqMan DNA minor groove-binding probes with FAM or VIC dye for detection and two PCR primers. Assay IDs for the genes examined were as follows: *Th* (Mm00447546_m1), *Vmat2* (Mm00553058_m1), *Dat* (Mm00438388_m1), *Da-d2r* (Mm00438545_m1), *Pitx3* (Mm00435722_m1), *Nurr1* (Mm00443060_m1), and *GAPDH* (4352339E). Each assay was optimized by universal thermal cycling conditions with a final reaction concentration of 250 nM for the probe and 900 nM for each primer. The cDNA (0.5 μl, 1/10 dilution) was mixed with H_2O and 2× TaqMan Universal PCR Mix (Applied Biosystems). The total reaction volume was 15 μl and the reactions were performed in triplicate. The thermal cycling conditions were as follows: 50 °C (2 min) and 95 °C (10 min), followed by 40 cycles at 97 °C (15 s) and 60 °C (1 min). Rotor-Gene's software and relative quantification against calibrator samples were used for analysis. The relative fold changes were determined by the method of $2-\Delta\Delta Ct$ as described previously (Livak and Schmittgen, 2001).

Western blot analysis

Additional groups were used as described above ($n = 4$ animals per group) to analyze Th and Nurr1 proteins in the striatum and midbrain. Th and Nurr1 proteins were analyzed from cytoplasmic and nuclear extracts, respectively. Cytoplasmic and nuclear extracts were prepared using Dignam's method (1983) with modifications (Ojeda et al., 2003). Briefly, the striatum or midbrain was dissected and gently homogenized in 0.45 vol buffer A. Buffer A contains 20 mM Hepes, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1 mM Dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF). Following 2 min centrifugation at 14,000*g*, the supernatant was saved as the cytoplasmic extract. The pellet was then resuspended in buffer C (20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EGTA, 20% glycerol, 0.42 M KCl, 1 mM DTT, and 1 mM PMSF). The homogenized tissue samples were kept on ice for 30 min with agitation. Finally, the suspension was centrifuged at 14,000*g* for 20 min, and the supernatant was saved as the nuclear extract. The protein concentrations were determined by the Bradford method (Bradford, 1976).

Samples containing 30 μg of protein were boiled for 10 min and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12%). They were then blotted onto nitrocellulose membranes and blocked by immersion in PBS-Triton 1% containing 5% milk for 2 h at room temperature. Immunodetection was performed at 4 °C overnight with the respective primary antibodies: anti-Th (1:500, MAB5280; Chemicon, Temecula, CA, USA), anti-Nurr1 (1:500, sc-991; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti β-actin (1:500, sc-130657; Santa Cruz Biotechnology), and anti-TATA box-binding protein (TBP) (1:1000, 05-1531; Chemicon International). After rinsing three times in PBS-triton 1% for 10 min, blots were incubated for 1 h at room temperature with a secondary horseradish peroxidase-conjugated antibody (1:2000, sc-2004 and sc-2031; Santa Cruz Biotechnology). Immunoreactivity was visualized by an ECL fluorescence detection system (GE Healthcare, Piscataway, NJ, USA). The intensity of the protein bands was measured by densitometry. The results were expressed as a ratio to β-actin (cytoplasmic extract) or TBP (nuclear extract) intensity to ensure correct measurement.

Statistical analyses

The data are expressed as the means ± standard errors. All data were analyzed using one-way analyses of variance (ANOVA), followed by *post hoc* Duncan's tests. Values of $p < 0.05$, $p < 0.01$ and $p < 0.001$ were considered to be statistically significant. Statistical analyses were performed using SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA).

RESULTS

Changes in gene expression in the midbrain

In the present study we analyzed the effects of EGb 761 treatment on DA-related genes under MPTP neurotoxicity. EGb 761 administration to control mice (''saline + EGb 761'' group) did not produce changes in gene expression (*Th*, *Vmat2*, *Dat*, *Da-d2r*, *Pitx3*, and *Nurr1*) when compared to the ''saline + saline'' group (Figs. 1A, 2A–B, 3A).

Th is the initial and rate-limiting enzyme in the synthesis of DA (Goridis and Rohrer, 2002). We found that the relative mRNA levels of *Th* were reduced (51%) in the midbrains of MPTP-lesioned mice compared to control animals (''saline + saline'') (Fig. 1A). This finding is the result of the neurotoxic action of MPTP on the DA system. This reduction of *Th* mRNA could be reversed by EGb 761 administration (41%) to the MPTP-treated group.

The changes in *Th* mRNA levels were paralleled by similar effects in protein levels (Fig. 1B, C) as measured by Western blot analysis. We found that Th protein levels were significantly decreased in the midbrain of mice treated with MPTP (28%). This reduction was partially reversed by EGb 761 (34%) when administered to the MPTP group.

Both Vmat2 and Dat are phenotypic markers for mature DA neurons. The expression profile of *Vmat2* mRNA in the midbrain (Fig. 2A) produced by MPTP showed a reduction in its gene expression (27%). However, EGb 761 produced partial protection (15%) of *Vmat2* mRNA in MPTP-treated mice. Furthermore, mice in the MPTP group presented markedly reduced (70%) *Dat* mRNA levels. The administration of EGb 761 to MPTP-treated mice increased (102%) *Dat* mRNA levels (Fig. 2A).

Dopamine D2 receptor (Da-d2r) is a major target for PD treatment and is important for DA signaling pathways (Bonci and Hopf, 2005). The MPTP-treated group displayed a significant reduction in *Da-d2r* mRNA (36%) as a result of dopaminergic neurotoxicity (Fig. 2B). In contrast, EGb 761 treatment significantly protected *Da-d2r* mRNA (46%) in the MPTP group (Fig. 2B).

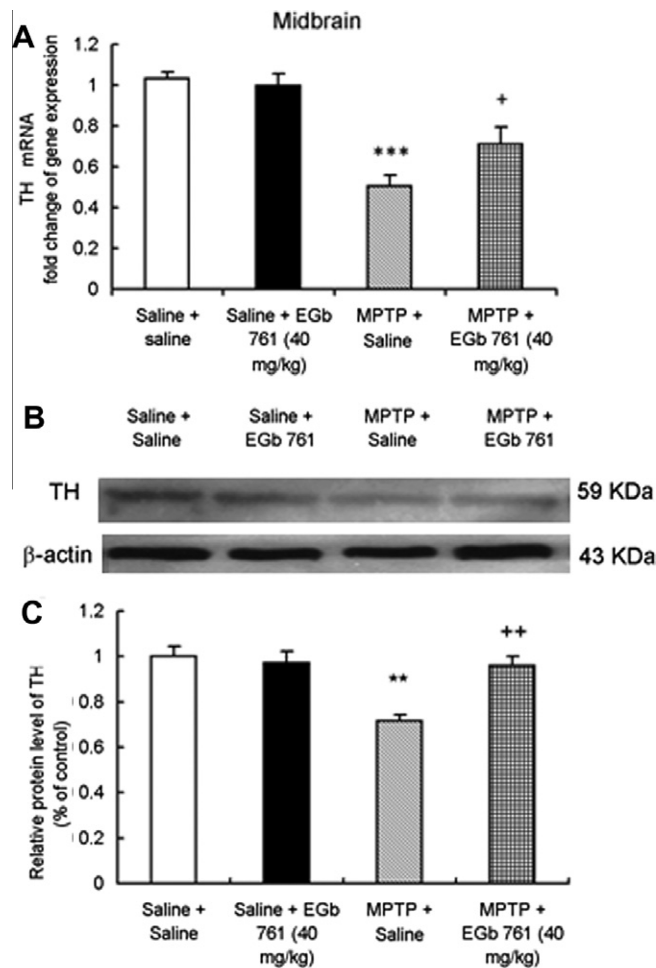

Fig. 1. EGb 761 upregulates *Th* mRNA and protein levels against the dopaminergic neurotoxicity induced by MPTP in the midbrain. (A) Relative *Th* mRNA levels were analyzed by qPCR. The expression levels were normalized to the *GAPDH* gene. The results are expressed as fold change compared to the control group (saline + saline) of 6–8 animals per group. (B) Representative Western blot of Th and β-actin (C) Densitometric analysis of Western blot of Th protein levels in midbrain compared to β-actin ($n = 4$ animals per group). Data are expressed as the mean ± SEM. Differences were analyzed with a one-way ANOVA followed by *post hoc* Duncan's tests $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the "saline + saline" group; $^{+}P < 0.05$ and $^{++}P < 0.01$ compared to the "MPTP + saline" group. MPTP, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine; EGb 761, *Ginkgo biloba* extract.

Pitx3 is a transcription factor that is expressed almost exclusively in midbrain DA neurons. The loss of *Pitx3* expression causes a reduction in substantia nigra dopaminergic neurons (Nunes et al., 2003). Mice in the MPTP group presented a marked reduction (73%) in *Pitx3* mRNA levels (Fig. 2B). However, EGb 761 administration to MPTP-treated mice enhanced (63%) *Pitx3* mRNA levels.

Nurr1 is a critical transcription factor for the maintenance of adult DA neurons (Jankovic et al., 2005) and DA neurotransmission. The expression of *Nurr1* (Fig. 3A) was significantly reduced (74%) in the MPTP-treated group compared to vehicle-treated controls. This reduction in *Nurr1* mRNA was reversed by EGb 761 administration (148%) to the MPTP-treated group. To correlate the enhancement of *Nurr1* mRNA with that of the Nurr1 protein, Western blot analysis was performed. Because Nurr1 is a transcription factor, the nuclear extract obtained from the midbrain was analyzed. As shown in Fig. 3B, C, a clear band with an approximate molecular weight of 66 kDa corresponding to the Nurr1 protein was detected. We also found that the Nurr1 protein levels were significantly decreased (37%; 3B-C) in the midbrain of MPTP-treated mice. This reduction in Nurr1 protein was partially reversed by EGb 761 (39%) in the MPTP group.

Changes in gene expression in the striatum

EGb 761 administration to control mice ("saline + EGb 761" group) did not produce changes in gene expression (*Th*, *Vmat2*, *Dat*, *Da-d2r*, *Pitx3*, and *Nurr1*) when compared to the "saline + saline" group (Figs. 4A, 5A–B, 6A). However, we found that the relative mRNA levels of *Th* were reduced (46%; Fig. 4A) in striatum of MPTP-lesioned mice when compared to control animals ("saline + saline") (Fig. 4A). This was a result of the neurotoxic action of MPTP in the DA system. The reduction of *Th* mRNA could be partially reversed by EGb 761 administration (48%) to the MPTP-treated group.

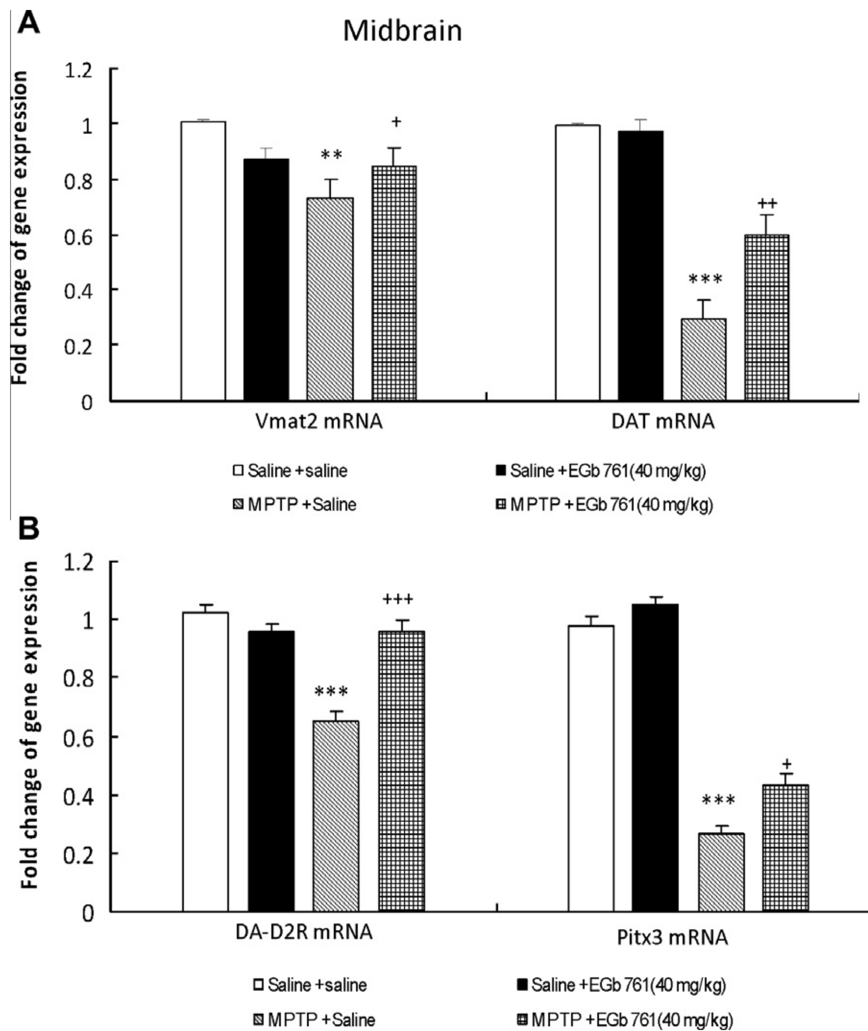

Fig. 2. EGb 761 upregulates the expression of dopamine-related genes against dopaminergic neurotoxicity produced by MPTP in the midbrain. Quantification of mRNA by qPCR of (A) *Vmat2*, and *Dat*, (B) *Da-d2r* and *Pitx3*. The expression levels were normalized using the *GAPDH* gene. The results are reported as fold change compared to the control group (saline + saline). Six to eight mice were used per group; values are the mean ± SEM. Differences were analyzed with one-way ANOVA followed by *post hoc* Duncan's tests. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to "saline + saline" group; $^{+}P < 0.05$, $^{++}P < 0.01$ and $^{+++}P < 0.001$ compared to "MPTP + saline" group. MPTP, 1-Methyl-4-phenyl-1,2,3, 6-tetrahydropyridine; EGb 761, *Ginkgo biloba* extract.

The changes in *Th* mRNA levels were paralleled by similar effects in protein levels (Fig. 4B, C) as measured by Western blot analysis. We found that the Th protein levels were significantly decreased in the striatum of mice treated with MPTP (25%). This reduction was partially reversed by EGb 761 (24%) administered to the MPTP group. No changes were found in the *Vmat2*, *Dat*, or *Da-d2r* expression levels across any of the experimental groups (Fig. 5A, B).

In contrast, mice in the MPTP group showed a considerable reduction in the level of *Pitx3* mRNA expression (57%; Fig. 5B). The administration of EGb 761 to MPTP-treated mice did not produce differences in *Pitx3* mRNA levels.

Nurr1 gene expression was significantly reduced (68%; Fig. 6A) in the MPTP-treated group compared to the vehicle-treated control. This reduction in *Nurr1* mRNA was reversed by EGb 761 administration (96%) to the MPTP-treated group. To correlate the enhancement of *Nurr1* mRNA with Nurr1 protein, a Western blot analysis was performed from the nuclear extract obtained from the striatum. As shown in Fig. 6B, C a clear band with an approximate molecular weight of 66 kDa corresponding to the Nurr1 protein was obtained. We found that the level of Nurr1 protein was significantly decreased in the striatum (34%) of mice treated with MPTP. This reduction in Nurr1 protein was partially reversed by EGb 761 (27%) in the MPTP group.

DISCUSSION

In the present study we report that the neuroprotective effect of EGb 761 in MPTP neurotoxicity is through the modulation of the expression of DA-related genes. These genes included *Th*, *Vmat2*, *Dat*, *Da-d2r*, and transcription factors related to the DA phenotype (*Pitx3* and *Nurr1*).

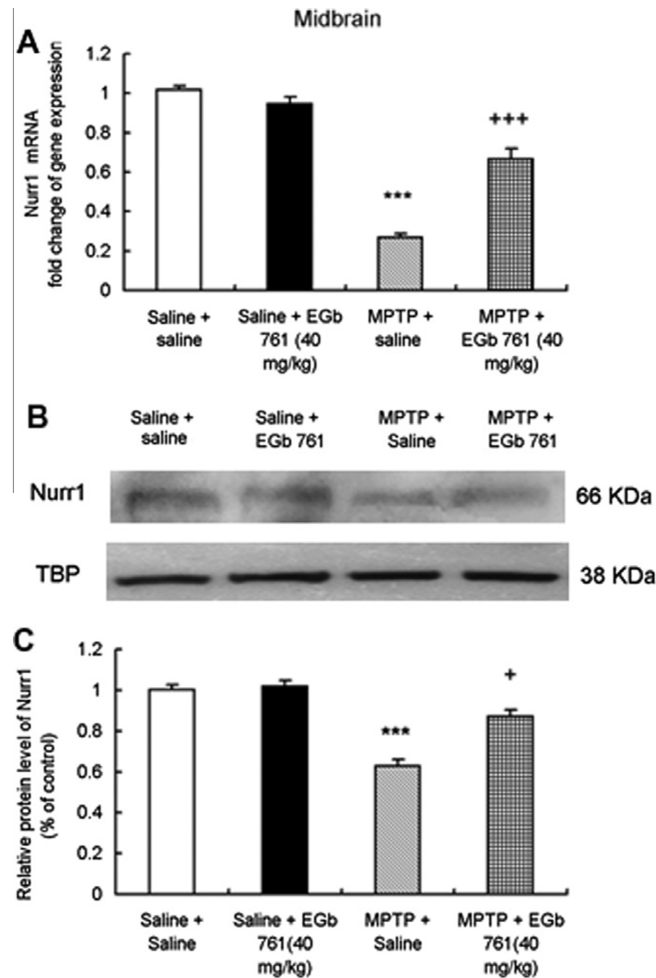

Fig. 3. Upregulation of *Nurr1* gene expression and protein levels by EGb 761 against MPTP-induced neurotoxicity in the midbrain. (A) Relative levels of *Nurr1* mRNA were analyzed by qPCR. The expression levels were normalized using the GAPDH gene. The results are reported as fold change compared to the control group (saline + saline) of 6–8 animals per group. (B) Representative Western blot of Nurr1 and TBP proteins. (C) Densitometric analysis of Western blot of Nurr1 protein levels in midbrain compared to TBP protein ($n = 4$ mice per group). Data are expressed as the mean ± SEM. Differences were analyzed with one-way ANOVA followed by *post hoc* Duncan's tests. $^{***}P < 0.001$ compared to the "saline + saline" group; $^{+}P < 0.05$ and $^{+++}P < 0.001$ compared to the "MPTP + saline" group. TBP, TATA box-binding protein; MPTP, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine; EGb 761, *Ginkgo biloba* extract.

In particular, we used a standardized extract of *G. biloba* named EGb 761 (Drieu, 1986). EGb 761 is used clinically for the treatment of neurological diseases related to the production of free radicals that lead to oxidative stress (DeFeudis and Drieu, 2000). The isolated constituents of EGb 761 have been found to be potent antioxidants (Droy-Lefaix et al., 1995; Guidetti et al., 2001) and free radical scavengers (Maitra et al., 1995; Ni et al., 1996). The therapeutic benefits of this extract may reside in the synergistic effect of all characterized components (DeFeudis, 1998).

We have previously reported the importance of EGb 761 as a therapeutic alternative in the future treatments of PD (Rojas et al., 2012). Our studies support that EGb 761 can provide effective neuroprotection of striatal DA content compromised by MPTP (Rojas et al., 2008). In that study, EGb 761 also improved the MPTP-induced impairment of locomotion in a manner that correlated with an enhancement of striatal DA content. We have also reported that EGb 761 regulates MAO activity and enhances Th content in MPTP neurotoxicity (Rojas et al., 2004). This finding shows that the EGb 761 neuroprotective effect is, in part, acting on the DA system. Therefore, we examined whether EGb 761 exerts dopaminergic neuroprotection through the regulation of DA-related gene expression that is altered by MPTP neurotoxicity. In the current study, we found that EGb 761 is able to protect gene expression against MPTP neurotoxicity, indicating an upregulation of DA-related genes.

The ability of EGb 761 to affect gene expression provides a novel mechanistic perspective regarding its biological activity. For example, EGb 761 influences proliferation, differentiation, apoptosis, and genes associated with stress and antioxidant protection (DeFeudis, 2002; Gohil and Packer, 2002; Smith and Luo, 2004). Indeed, the levels of mRNAs associated with various transcription factors, antioxidant enzymes, DNA synthesis and repair are upregulated by EGb 761 treatment (DeFeudis, 2002). There are several genes

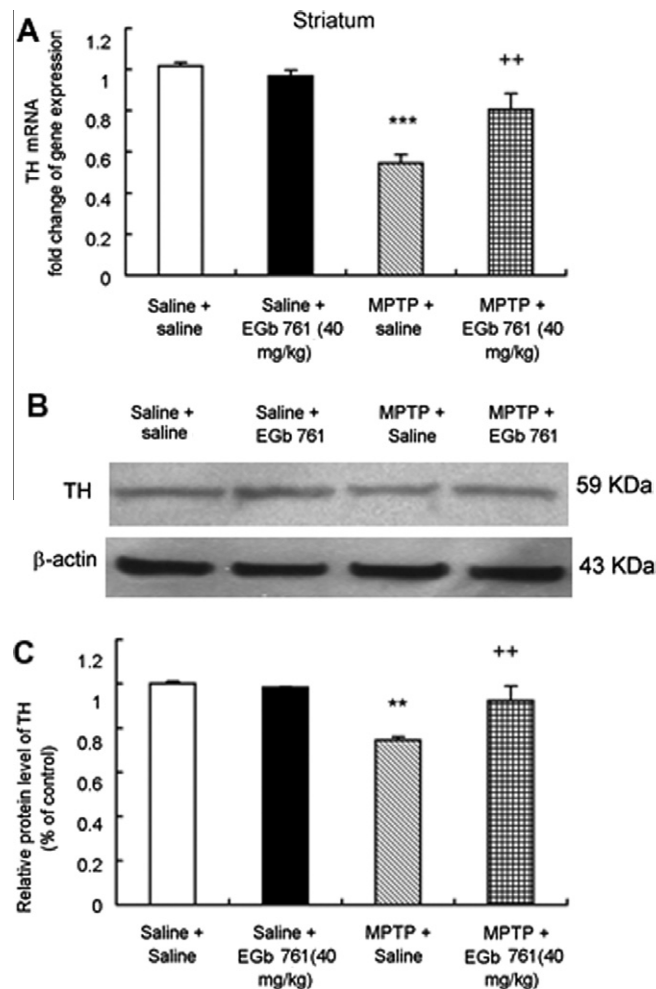

Fig. 4. EGb 761 upregulates *Th* mRNA and protein levels against MPTP-induced neurotoxicity in the striatum. (A) Quantification of *Th* mRNA by qPCR. The levels of expression were normalized using the *GAPDH* gene. The results are reported as fold change compared to the control group (saline + saline) of 6–8 animals per group. (B) Representative Western blot of Th and β-actin protein. (C) Densitometric analysis of Western blot of Th protein levels in striatum as compared to β-actin ($n = 4$ mice per group). Data are expressed as the mean ± SEM. Differences were analyzed with one-way ANOVA followed by *post hoc* Duncańs tests. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the ''saline + saline'' group; $^{++}P < 0.01$ compared to the ''MPTP + saline'' group. MPTP, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine; EGb 761, *Ginkgo biloba* extract.

whose expression is important for the function of DA neurons. Furthermore, it has been reported that gene expression related to DA neurotransmission is decreased in PD (Harrington et al., 1996; Nagatsu and Sawada, 2007; Liu et al., 2012). The study of gene expression is important because it could be a potential biomarker in the preclinical diagnosis of PD.

It has been reported that MPTP decreases the expression of *Th*, *Dat*, *Vmat2* (Xu et al., 2005), and *Nurr1* (Gibrat et al., 2009). The present study suggests that the neuroprotective effect of EGb 761 against MPTP neurotoxicity involves the modulation of gene expression.

The first enzyme involved in the formation of DA is the rate-limiting enzyme Th, which converts the amino acid tyrosine into levodopa. In particular, TH activity, TH synthesis, and *TH* mRNA levels are markedly decreased in the striatum and substantia nigra of PD patients (Nagatsu and Sawada, 2007). In the current study we found that EGb 761 protected against the reduction of *Th* mRNA in the nigrostriatal pathway that is produced by MPTP neurotoxicity. The upregulation of *Th* mRNA levels was paralleled by similar effects on Th protein levels. This effect could be the result of an enhancement of its transcription that could increase DA synthesis. Consistent with this, we previously reported that EGb 761 upregulates Th activity in conditions of $MPTP/MPP^+$ neurotoxicity (Rojas et al., 2004).

Once DA is synthesized it is stored in synaptic vesicles by Vmat2. A reduction in *VMAT2* expression has been reported in PD patients (Frey et al., 2001). Furthermore, *Vmat2* mRNA levels were found to be reduced in the platelets (Sala et al., 2010) and substantia nigra of PD patients (Harrington et al., 1996). These findings suggest the existence of an impairment of this transporter that could contribute to PD pathology (Sala et al., 2010). Moreover, polymorphisms in the gene that encodes VMAT2 have been identified (Glatt et al., 2008). It has been suggested that some polymorphisms could affect *VMAT2* expression and might be associated with an increased risk for PD. We found that MPTP produces a reduction in the

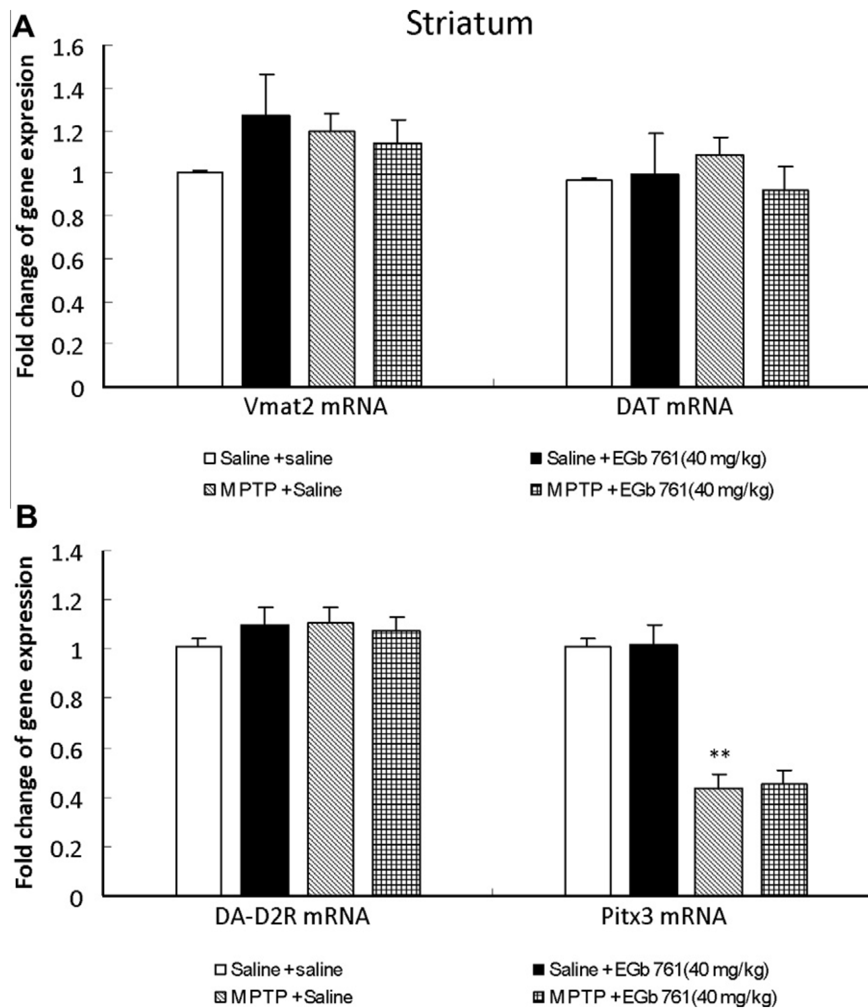

Fig. 5. Effect of EGb 761 on dopamine-related gene mRNA against MPTP dopaminergic neurotoxicity in the striatum. Quantification of mRNA by qPCR of (A) *Vmat2* and *Dat and* (B) *Da-d2r* and *Pitx3.* The expression levels were normalized using the *GAPDH* gene. The results are reported as fold change compared to the control group (saline + saline). Six to eight mice were used per group, values are the mean ± SEM. Differences were analyzed with one-way ANOVA followed by *post hoc* Duncan's tests. $^{**}P < 0.01$ compared to the "saline + saline" group. MPTP, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine; EGb 761, *Ginkgo biloba* extract.

expression of *Vmat2* mRNA as previously reported (Jourdain et al., 2005). We also showed that EGb 761 protects *Vmat2* mRNA expression in MPTP-treated mice in the cell body region of the nigrostriatal pathway located in the midbrain.

DA neurotransmission is terminated by uptake of DA into presynaptic terminals by Dat. A reduction in *DAT* mRNA has been reported in PD patients (Harrington et al., 1996). This reduction was associated with a marked loss of DA-containing cells in the substantia nigra. Moreover, reduced *DAT* expression strongly correlates with the extent of DA cell loss in PD (Panzacchi et al., 2008). In addition, allelic variants in the *DAT* gene that affect gene expression are associated with an increased risk for PD (Ritz et al., 2009). These data suggest that a polymorphism in the genes involved in DA regulation may modulate the susceptibility to PD (Singh et al., 2008). We found that MPTP reduces the expression of *Dat* mRNA in the midbrain as previously reported (Miller et al., 2004). The administration of EGb 761 to MPTP-treated mice produced an enhancement in *Dat* mRNA expression. This finding demonstrates an upregulation of *Dat* mRNA by this natural extract in conditions of MPTP neurotoxicity.

Upon its release from presynaptic terminals into the synaptic cleft, DA exerts its effect by interacting with presynaptic or postsynaptic DA receptors generating the intracellular signals in the neurons. DA-D2R is a major target of PD treatment and is an important player in the DA signaling pathways (Bonci and Hopf, 2005). In particular, DA-D2R is involved in the regulation of DA synthesis, metabolism and release. A decline in DA-D2R was observed in late-stage PD (Rinne et al., 1991). Furthermore, polymorphism of *DA-D2R* gene was shown to be associated with low DA-D2R density in humans (Pohjalainen et al., 1998). Thus, differences in the expression of the *DA-D2R* gene might affect DA response, determining a genetic predisposition to PD (Costa-Mallen et al., 2000).

Previous studies have reported that the MPTP administration produces a reduction in Da-d2r in the midbrain (Araki et al., 2001). We found that the

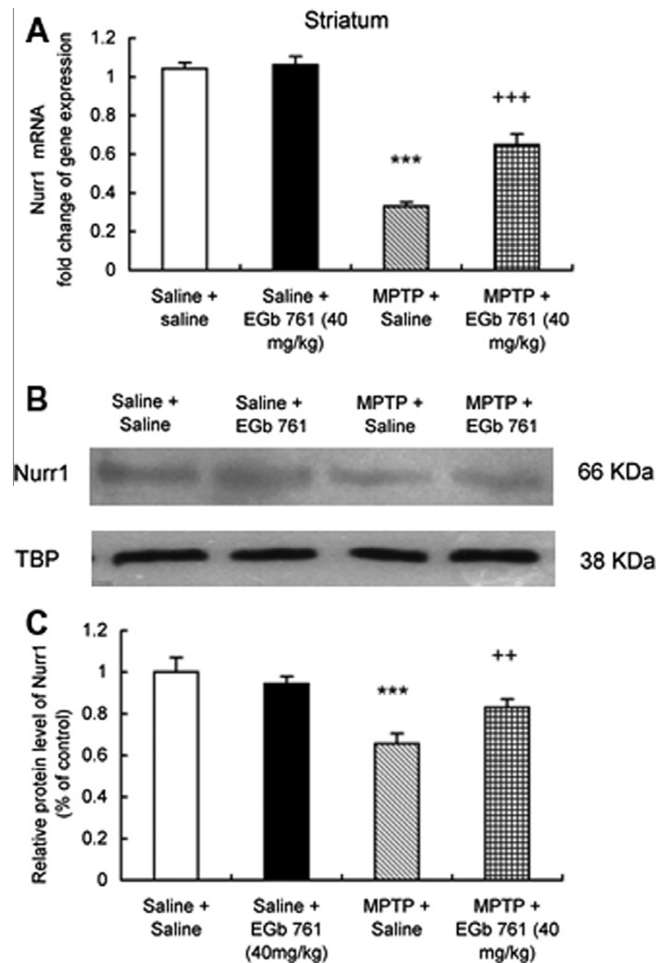

Fig. 6. Upregulation of *Nurr1* mRNA and protein levels by EGb 761 against dopaminergic neurotoxicity induced by MPTP in the striatum. (A) The relative Nurr1 mRNA levels were analyzed by qPCR. The expression levels were normalized using the *GAPDH* gene. The results are reported as fold change compared to the control group (saline + saline). (B) Relative Western blot of Nurr1 and TBP protein in striatum. (C) Densitometric analysis of Western blot of Nurr1 protein levels in striatum compared to TBP protein ($n = 4$ mice per group). Data are expressed as the mean ± SEM. Differences were analyzed with one-way ANOVA followed by *post hoc* Duncan's tests. $^{***}P < 0.001$ compared to the ''saline + saline'' group; $^{++}P < 0.01$ and $^{+++}P < 0.001$ compared to the ''MPTP + saline'' group. TBP, TATA box-binding protein; MPTP, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine; EGb 761, *Ginkgo biloba* extract.

expression of *Da-d2r* mRNA was reduced in conditions of MPTP neurotoxicity and was increased in EGb 761-treated mice exposed to MPTP in the midbrain. This result shows that a modulation in gene expression is produced by EGb 761 in the cell body region of the dopaminergic nigrostriatal pathway. However, no changes in *Da-d2r* mRNA expression were found as a result of MPTP neurotoxicity in the striatum. This effect could be related to the heterogeneity in the responsiveness of the two analyzed neuroanatomical regions. This differential action produced by EGb 761 was also reported in other brain regions (Watanabe et al., 2001; Su et al., 2009). In addition, the nigrostriatal pathway is a specific target of EGb 761 (DeFeudis and Drieu, 2000) with a different response profile in each brain region.

The transcription factor Pitx3 is expressed almost exclusively in midbrain DA neurons (Smidt et al., 1997), and plays a crucial role in the maturation and survival of DA neurons, suggesting an association with PD pathogenesis. The loss of its expression results in a reduction in midbrain DA neurons (Nunes et al., 2003); it has been reported that *PITX3* gene expression is reduced in PD patients (Liu et al., 2012). Polymorphisms in *PITX3* are associated with sporadic and early onset PD (Li et al., 2009). We found that EGb 761 administration to MPTP-treated mice produced an upregulation of *Pitx3* and *Th* gene expression in the midbrain. Their relationship is supported as Pitx3 has been related to *Th* expression (Messmer et al., 2007); the promoter region of *Th* has potential binding elements for Pitx3 (Messmer et al., 2007).

Nurr1 is a transcription factor essential for the generation and functional maintenance of differentiated dopaminergic neurons (Chung et al., 2002). Previous studies have shown that *NURR1* gene expression is reduced in PD patients (Liu et al., 2012). Furthermore, *NURR1* mutations have resulted in a marked decrease in *NURR1* mRNA levels in PD (Le et al., 2003). It has also been reported that abnormalities in the *NURR1*

gene might be a risk factor in sporadic and familial PD (Xu et al., 2002). Moreover, reduced expression of *Nurr1* increases the sensitivity of dopaminergic neurons to MPTP neurotoxicity (Le et al., 1999). These findings suggest that NURR1 could be a good biomarker for PD.

In this study we investigated the expression of *Nurr1* mRNA and Nurr1 protein in the nigrostriatal pathway. We observed that *Nurr1* mRNA expression was significantly reduced in the MPTP-treated group as previously reported (Gibrat et al., 2009). However, this reduction in *Nurr1* mRNA expression was reversed by EGb 761 administration to the MPTP-treated group. Our study also showed that MPTP produces a reduction of Nurr1 protein, and this effect was reversed by EGb 761. However, EGb 761 protects the levels of Nurr1 protein in MPTP neurotoxicity. *Nurr1* is an immediate early gene that codes for a transcription factor. Accordingly, EGb 761 has been shown to affect the expression of the immediate early gene, *c-fos* (Lee et al., 2009) and other transcription factors (Soulié et al., 2002) as shown in the current study.

Nurr1 is able to regulate the expression of *Th*, *Vmat2*, *and Dat* (Jankovic et al., 2005; Smidt and Burbach, 2007), which could lead to an increase in DA. Our study shows that EGb 761 is able to upregulate the mRNA expression of *Th*, *Vmat2*, *Dat*, *Da-d2r*, *Pitx3*, and *Nurr1* in MPTP neurotoxicity.

The enhanced expression of *Pitx3* and *Nurr1* contributes to the regulation of the expression of DA-related genes, resulting in the production of DA neurotransmitter. These transcription factors might be related to the neuroprotective effect produced by EGb 761 in MPTP neurotoxicity.

We reported that EGb 761 is able to protect against dopaminergic neurodegeneration produced by MPTP (Rojas et al., 2008). The current study shows that one possible mechanism of this neuroprotection is related to the regulation of the DA genes. Previous studies have also reported that EGb 761 provides neuroprotection through the regulation of several genes in different neurodegenerative animal models (Augustin et al., 2009; Mahdy et al., 2011). Furthermore, its neuroprotection also involves the regulation of transcription factors related to dopaminergic neurons in an animal model of PD.

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