Research report

Neuroprotective effects of endurance exercise against neuroinflammation in MPTP-induced Parkinson's disease mice

Yongchul Jang^ac, Jung-Hoon Koo^ab, Insu Kwon^ab, Eun-Bum Kang^a, Hyun-Seob Um^b, Hideaki Soya^c, Youngil Lee^d, Joon-Yong Cho^a,⁎

^a Exercise Biochemistry Laboratory, Korea National Sport University, 88-15 Oryun-dong, Songpa-gu, Seoul 138-763, Republic of Korea
^b Department of Exercise Prescription, Kon-Yang University, 119 Daehangro, Nonsan city, Chungnam 320-711, Republic of Korea
^c Laboratory of Exercise Biochemistry and Neuroendocrinology, Faculty of Health and Sports Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8574, Japan
^d Exercise Biochemistry Laboratory, University of West Florida, 11000 University Pkwy, Bldg. 72, Pensacola, FL 32514, USA

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ABSTRACT

Parkinson's disease (PD) is one of the main degenerative neurological disorders accompanying death of dopaminergic neurons prevalent in aged population. Endurance exercise (EE) has been suggested to confer neurogenesis and mitigate the degree of seriousness of PD. However, underlying molecular mechanisms responsible for exercise-mediated neuroprotection against PD remain largely unknown. Given the relevant interplay between elevated α-synuclein and neuroinflammation in a poor prognosis and vicious progression of PD and anti-inflammatory effects of EE, we hypothesized that EE would reverse motor dysfunction and cell death caused by PD. To this end, we chose a pharmacological model of PD (e.g., chronic injection of neurotoxin MPTP). Young adult male mice (7 weeks old) were randomly divided into three groups: sedentary control (C, n=10), MPTP (M, n=10), and MPTP + endurance exercise (ME, n=10). Our data showed that EE restored motor function impaired by MPTP in parallel with reduced cell death. Strikingly, EE exhibited a significant reduction in α-synuclein protein along with diminished pro-inflammatory cytokines (i.e., TNF-α and IL-1β). Supporting this, EE prevented activation of Toll like receptor 2 (TLR2) downstream signaling cascades such as MyD88, TRAF6 and TAK-1 incurred by in MPTP administration in the striatum. Moreover, EE reestablished tyrosine hydroxylase at levels similar to C group. Taken together, our data suggest that an EE-mediated neuroprotective mechanism against PD underlies anti-neuroinflammation conferred by reduced levels of α-synuclein. Our data provides an important insight into developing a non-pharmacological countermeasure against neuronal degeneration caused by PD.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder affecting 1–2% of the elderly population and is characterized by cognitive deficits and impaired motor functions (Petrzinger et al., 2013). These impairments stem from the progressive loss of dopaminergic neuronal cells in the substantia nigra par compacta, resulting in the depletion of dopamine in the striatum. The decreased levels of dopamine in the striatum are responsible for a motor impairment observed in PD patients (Bezard et al., 2013). Currently, five genes (α-synuclein, parkin, dj-1, pink1, and lrrk2) have been identified to cause PD (Thomas and Beal, 2007). Among these is α-synuclein (α-Syn) a key factor in the pathogenesis of PD based on genetic, neuropathological, and cellular/molecular lines of evidence, suggesting that elevated levels of α-Syn exert deleterious effects on dopaminergic neurons (Lim et al., 2003). Also, postmortem investigations of PD and other α-synapathies studies have demonstrated the relevance of oligomeric α-Syn aggregates to PD (Spillantini et al., 1997; Spillantini and Goedert, 2000).

Consequence of accumulation of oligomeric α-Syn in neurons has been implicated in activating a serious of Toll-like receptor-2 (TLR2)-mediated signaling cascades through myeloid differentiation factor-88 and nuclear transcription factor-xB (NF-xB), triggering the cerebral inflammation (Codoło et al., 2013; Drouin-Ouellet et al., 2011; Hayward and Lee, 2014; Kim et al., 2013). Indeed, multiple lines of studies have confirmed that the recognition of α-Syn by TLR2 results in production of pro-inflammatory cytokines TNF-α and IL-1β that are toxic and thus contribute to cell death of dopaminergic neurons (Eikelenboom et al., 2006; Hensley et al., 2006). These studies suggest

⁎ Corresponding author.
E-mail address: chojy86@knsu.ac.kr (J.-Y. Cho).

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that a potential strategy to promote anti-inflammatory countermeasure may significantly contribute to prevention of PD.

Endurance exercise has emerged as a potent, non-pharmacological intervention in the management of patients with PD. A large number of studies in neuroscience have shown that physical exercise improves the symptoms and progression of PD, suggesting exercises as a potential preventive countermeasure against PD (Al-Jarrah et al., 2013; Petzinger et al., 2007; Ridgel et al., 2009). Relating to this, growing evidence indicates that exercise-mediated neuroprotective effects against PD associate with reduced cerebral inflammation (Spelman et al., 2016). Currently, however, how exactly endurance exercise induces anti-inflammatory effects at molecular levels. In the present study, we induced PD-like symptoms in the mice brain via the administration of MPTP, after which 8 weeks of treadmill endurance running exercise were incorporated to test our research hypothesis that endurance exercise-induced decline in α-Syn would prevent activation of TLR2-mediated neuroinflammation.

We report here that endurance exercise restores MPTP-induced motor impairments in parallel with reestablishment of dopamine and prevention of cell death and apposes the production of proinflammatory cytokines by modulating TLR2/MyD88/NF-κB activation. Taken together, our data suggest that endurance exercise-induced neuroprotection against PD may be implicated in downregulation of α-Syn, subsequently leading to anti-inflammation and anti-cell death.

2. Results

2.1. Endurance exercise restores motor coordination deficits caused by MPTP-induced PD via preventing the accumulation of α-Syn

Key manifestation of PD is a gradual loss of motor function. To examine if EE prevents PD-induced motor coordination impairment, endurance exercise was introduced to MPTP-induced mice for 8 weeks, and retention time assessed via the rota-rod test was measured. MPTP-induced PD mice exhibited significant reduction in the retention time compared with saline-treated control mice; however, endurance exercise-trained MPTP-mice completely abolished the MPTP-induced decline of retention time relative to MPTP-mice (Fig. 1A and B). Given the fact that aberrant accumulation of α-Syn is linked to motor function deficit, we examined if exercise-mediated improvement of motor coordination in MPTP-mice is associated with modulation of α-Syn. Our western blot (Fig. 1C and D) and immunofluorescence microscopy (Fig. 1E) data showed that endurance exercise prevented MPTP-induced elevation of α-Syn.

2.2. Endurance exercise mitigates TLR2 activation in the brain of MPTP-induced PD mice

In order to examine our hypothesis that EE-induced ablation of α-Syn upregulation caused by MPTP-treatment would mitigate neuroinflammation, we assessed levels of a key initiator of inflammation TLR2. Our western blot and immunofluorescence microscopy data showed that EE prevented MPTP-induced elevation of striatum TLR2, whereas MPTP-treated sedentary control mice displayed significant elevation of TLR2 (Fig. 2A, B and F). Similarly, EE prohibited MPTP-induced upregulation of MyD88, a downstream molecule of TLR2 (Fig. 2A and C) and tumor necrosis factor receptor-associating factor 6 (TRAF6), a downstream target of MyD88 (Fig. 2A and D), compared with MPTP treatment. Subsequently, EE inhibited MPTP-mediated activities of transforming growth factor-β-activated protein kinase 1 (TAK1), a downstream signaling molecule of TRAF6 inactivation (phosphorylation) of (Fig. 2A and E).

2.3. Endurance exercise reverses MPTP-induced proinflammatory cytokine overproduction and prevents apoptotic cell death in striatum

Active (phosphorylated) NF-κB via phosphorylated IκBα translocates to nucleus and initiate the expression of proinflammatory cytokines TNF-α and IL-1β. While MPTP-treated mice showed a marked elevation of both IκBα and NF-κB phosphorylation levels, those levels in ME mice remarkably declined to levels similar to control mice (Fig. 3A, B and C). Accordingly, ME mice showed reduced levels of TNFα and IL-1β compared to MPTP-mice (Fig. 3D and E). Finally, our immunohistochemical data confirmed that endurance exercise reversed apoptotic cell death caused by MPTP in the striatum (Fig. 3F).

2.4. Endurance exercise increases levels of striatum TH expression in MPTP-mice

In order to confirm whether opportune recovery of motor function by endurance exercise from MPTP-induced motor function impairment is due to enhanced dopamine production, we measured the levels of tyrosine hydroxylase (TH), an alternative measure of dopamine. TH levels remained repressed in the striatum of MPTP-treated mice whereas endurance exercise reinstated TH levels similar to control (Fig. 4A and B). Also, an immunostaining analysis confirmed the restoration of TH levels in striatum in response to endurance exercise after MPTP administration (Fig. 4C).

2.5. Endurance exercise augments levels of SNpc TH expression in MPTP-mice

To further confirm neuroprotective effects of endurance exercise in SNpc, we assessed levels of tyrosine hydroxylase (TH). Similar to Striatum, Western blot analysis showed that TH levels in SNpc were suppressed in MPTP-treated mice, but exercise training restored its level up to control group (Fig. 5A and B). Moreover, an immunohistochemical assessment showed an increase in numbers of TH positive neurons in exercise-trained SNpc, compared to MPTP group (Fig. 5C).

3. Discussion

The present study examined how endurance exercise (EE) provides neuroprotection using pharmacological model of Parkinson’s disease and demonstrated three key findings. First, EE restored motor performance capacity previously impaired by MPTP administration in parallel with suppressed α-Syn to a level comparable to non-PD control animals. Second, EE reversed PD-mediated neuroinflammation by hindering TLR2 downstream singling cascades. Finally, EE restored tyrosine hydroxylase to a level comparable to those displayed in control animals. Taken together, our results demonstrate that EE-induced neuroprotection against PD is mediated by α-Syn suppression, leading to anti-inflammatory effect via diminished TLR2 signaling. A detailed discussion of these findings follows.

A growing body of evidence has identified EE as a both preventive and therapeutic regimen in the management of patients with PD (Petzinger et al., 2013; Smith et al., 2011; Tuon et al., 2012). Our results that EE improves motor performance assessed by the rota-rod test in MPTP-treated mice are also consistent with the studies. However, exact molecular mechanisms responsible for exercise-mediated neuroprotection against PD are incompletely understood. Nevertheless, recent studies have implicated α-Syn downregulation via EE as a key factor linked to neuroprotection (Kohman et al., 2012; Petzinger et al., 2007; Yoon et al., 2007).

α-Syn is a monomeric protein necessary for proper synaptic process and transmission (Moore et al., 2005). However, aberrant accumulation of α-Syn in the dopamine-generating neurons is linked to neuroinflammation and cell death, leading to PD (Satiki, 2014). In the present study, we observed that long term MPTP administration, which
has been widely used to induce PD-like symptom, accumulates α-Syn in striatum and concurs with neuroinflammation. Although underlying mechanisms of MPTP-induced α-Syn accumulation is unknown, given that MPTP is associated with production of free radicals from mitochondria by inhibiting the complex I among electron transport chains, we presume that MPTP-induced oxidative stress may accelerate α-Syn gene expression.

Intriguingly, we found that endurance exercise reverses MPTP-induced α-Syn upregulation in striatum, similar to other studies showing downregulation of α-Syn by endurance exercise in different parts of the brain including hippocampus and cortex (Tsou et al., 2012). Although how exactly endurance exercise limits α-Syn upregulation remains undiscovered, and it is not researched in the present study, we propose on the basis of available body of literature three potential mechanisms that can describe exercise-induced α-Syn downregulation: 1) exercise-induced antioxidative capacity, 2) facilitated apoptosis whereby dying cells containing excessive α-Syn are quickly removed, and 3) enhanced autophagy and ubiquitin protease system that eliminates surplus α-Syn. Apparently, these mechanisms need to be elucidated in future studies.

Dysregulated neuroinflammation has emerged as a potent inducer of neuronal cell death (Spieldman et al., 2016). Toll like receptors (TLRs) are membrane-bounding proteins associated with immune system, recognizing their ligands and initiating a series of proinflammatory signaling pathways (Beraud and Maguire-Zeiss, 2012). Importantly, recent studies have suggested that α-Syn is a potential ligand of TLR2 and that interplay between α-Syn and TLR2 plays a critical role in the onset of neuroinflammation (Dimatélis et al., 2013; Flynn et al., 2007). Although we did not conduct immune-precipitation experiments to assess protein-protein interaction, we observed that endurance exercise downregulates both α-Syn and TLR2 in the striatum of MPTP-treated mice. This result suggests that exercise-mediated suppression of α-Syn and TLR2 may be an important element that hampers the interaction between α-Syn and TLR2. Supporting this, our results provide evidence that endurance exercise downregulates a series of TLR2 downstream signaling molecules; for example, levels of MyD88, an adaptor protein of TLR2, its downstream protein TRAF6, and the phosphorylation of TAK1 return back to the control level.

Nuclear translocation of NF-κB is necessary to initiate the expressions of pro-inflammatory cytokines, but this does not occur until its endogenous inhibitory unit IκBα dissociates from NF-κB (Gasparini and Feldmann, 2012). Multiple studies have demonstrated that the dissociation of IκBα from NF-κB happens in a phosphorylation dependent manner (Ferreiro and Komives, 2010); for example, phosphorylated IκBα is released from phosphorylated NF-κB and become the target of ubiquitin ligases for degradation. In line with these studies, our results show that MPTP treatment induces phosphorylation of cytosolic IκBα and facilitates nuclear translocation of NF-κB in parallel with upregulation of pro-inflammatory cytokines TNF-α and IL-1β, whereas endurance exercise reverses the consequence of MPTP treatment. The results indicate that blunting TLR2 signaling pathways via downregulating TLR2 expression is a key underlying mechanism of exercise-induced anti-inflammation. Postmortem studies have confirmed significant levels of apoptosis in the brain of PD patients and suggest apoptosis as a main cause of progressive loss of dopamine neurons in PD (Beraud and Maguire-Zeiss, 2012; Hanke and Kielian, 2011; Hayward and Lee, 2014; Kim et al., 2013). Since chronically elevated TNF-α and IL-1β are a well-accepted mechanism of death receptor-mediated intrinsic apoptosis (Montgomery and Bowers, 2012; Prajapati et al., 2015), prevention of TNF-α and IL-1β accumulation via EE may be critically linked to the protection against apoptotic cell death. Accordingly, our TUNEL assay results demonstrate that EE revokes apoptosis induced by MPTP treatment in the striatum.

In the present study, we observed low levels of apoptosis in the brain of exercise-trained animals that had developed PD like symptoms before exercise training was implemented. We believe that endurance exercise training-induced cell death prevention against MPTP treat-
ment may be due to enhanced apoptosis or other types of cellular debris removal systems such as autophagy even though we have not tested the postulation in the present study. In addition, based upon our previous study and recent emerging evidence acquired from animal and human studies, we propose that exercise-induced neurogenesis and neuro-restoration by promoting brain neurotrophic factors, synaptic strength, and angiogenesis may critically contribute to regeneration of neurons and thus restore normal motor function (Allen et al., 2015; Fisher et al., 2008; Marxreiter et al., 2013).

Accordingly, we investigated the effects of endurance exercise on viability of dopaminergic neurons in both striatum and SNpc of MPTP-treated mice because death of these neurons interrupts proper communication with cerebral cortex, leading to impairment in motor function (Cho et al., 2013; Sung et al., 2012; Yoon et al., 2007). Due to the limited sensitivity of mouse plasma dopamine, we instead assessed tyrosine hydroxylase (a rate-limiting enzyme involved in dopamine synthesis) using western blotting and immunohistochemistry and found that endurance exercise restores the TH levels in striatum and SNpc at levels equivalent to untreated controls. This observation suggests that endurance exercise may provide neuroprotection by replacing damaged or dead neurons with new neurons in striatum and SNpc, evidenced by the augmented TH levels in both tissues. Furthermore, we speculate that anti-inflammatory effects by endurance exercise (i.e., a reduced production of TNF-α and IL-1β and subsequent repression of TLR2) contribute to prevention of dopaminergic (DA) neuronal cell death.

In summary, our study suggests new evidence that endurance exercise-induced restoration of impaired motor function and cell viability caused by MPTP administration is linked to the repression of pro-inflammatory cytokines via downregulation of α-Syn and blunted TRL2 signaling pathways. This study provides a crucial insight into developing a potential non-pharmacological therapeutic countermeasure that improves PD.

4. Experimental method

4.1. Animals

All animal experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee at Korea National Sport University (KNSU-IACUC-2014-02). Thirty young adult (7-weeks old) male C57BL/6J mice were purchased from Samtako (Osan, Korea). The mice were maintained at a 12:12 h dark-light cycle, housed at 22 ± 2 °C with 50% relative humidity, and had free access to standard chow diet ad libitum.

4.2. MPTP-induced mouse model of Parkinson’s disease

To induce Parkinson’s disease, young adult (8 weeks old) male C57BL/6 J mice were injected with 25 mg/kg MPTP (Sigma-Aldrich,
St. Louis, MO) and 250 mg/kg probenecid (Sigma-Aldrich, St. Louis, MO) a total of 10 dose for 5 weeks by intraperitoneal injection, while control mice were treat with saline.

4.3. Endurance exercise

Mice (3 month-old) were divided into one of the following groups: control group (control, n=10), MPTP-induced group (M, n=10), and MPTP-induced endurance exercise group (ME, n=10). Animals assigned to ME were acclimated to treadmill running (Dae-myung Scientific Co, Ltd, Korea) at 8 m/min, 30 min/day for 5 days and performed endurance exercise training at 10 m/min, 60 min/day, 5 days/week on a 0% grade for 8 weeks. The endurance exercise has been successfully used in others and our previous studies (Guillot et al., 2008).

4.4. Rota-rod test as behavioral testing

Mice were introduced to walking on top of a rotating cylinder A rota-rod (JD-A-07RA5, Jeung Do Bio & Plant Co, Ltd), and walking speed was gradually increased every 30 s to evaluate their motor coordination function and sense of balance before and after experiment. The time when the animal fell off was recorded. Briefly, the mice were acclimated to the rotating cylinder moving at 10 rpm for 120 s.

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Fig. 3. The effects of endurance exercise on IκBα, NF-κB, TNF-α and IL-1β proteins and striatum neuronal apoptosis in MPTP-mice. (A) Representative Western Blot data (n=6). (B-E) Quantification of p-IκBα/t- IκBα, NF-κB, TNF-α and IL-1β proteins. β-actin serves as marker for equal loading. (F) TUNEL assay for neuronal apoptotic cells in the striatum (see arrows). Scale bars: 200 μm. Values are mean ± SEM.
Rota-rod tests were performed before and after 8 weeks of endurance exercise, with the speed increased by 5–40 rpm. Mice performed two sessions, and the average score of them was used. The maximum performance time was limited to 300 s.

### 4.5. Biochemical analysis and tissue preparation

Following the final round of exercise training and behavioral test, all mice were fasted for 24 h and anesthetized with 50 mg/kg of Zoletil50® (Virbac Laboratories, Carros, France) by intraperitoneal injection. Next, the brain tissues were removed. Then the striatum from hemispheres was isolated and then stored at −80 °C until needed. For immunohistochemistry and immunofluorescence staining, mice were perfused with 50 mM phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.4, after which brain tissues were collected and stored until needed.

### 4.6. Isolation of brain nuclear fraction

Nuclear fraction was prepared using a Subcellular Proteome Extraction Kit (Calbiochem, Darmstadt, Germany) according to the manufacturer’s instruction. In brief, the brain tissue was homogenized with 1 ml of extraction buffer I containing 5 μg of protease inhibitor cocktail and centrifuged at 1000×g for 10 min. The obtained pellet were gently resuspended, agitated in extraction buffer II containing 5 μg of protease inhibitor cocktail for 30 min on the rotary shaker, and then centrifuged at 6000g for 10 min. The obtained pellet were gently resuspended and agitated in 0.5 ml of Extraction buffer III containing 5 μg of protease inhibitor cocktail and 1.5 μg of Benzonase for 10 min on the rotary shaker. The final nuclear fraction was obtained by centrifugation at 7000×g for 10 min.

### 4.7. Western blotting

Western blot analyses were conducted as previously described (Um et al., 2011). Briefly, proteins (30 μg) were separated by 8–12% SDS PAGE, after which the proteins were transferred to a polyvinylidene fluoride membrane (PVDF) for 1 h at a constant voltage of 60 V. The membrane was then blocked with 5% BSA and incubated overnight at 4 °C with designated antibodies as follows: α-synuclein (BD biosciences, dilution, 1:1000); TH (Millipore, dilution, 1:1000); TLR2 (ab24192), TRAF6 (ab 3395), and MyD88 (abcam, dilution, 1:1000); NF-κBα (sc-4812), p-NF-κBα (sc-2859), TNF-α (sc-6956), IL-1β (sc-7884), β-actin (sc-47778), and Lamin B (sc-6216) antibodies (Santa Cruz, dilution, 1:1000). The membranes were then washed and incubated with secondary antibodies (HRP-conjugated goat anti-rabbit or rabbit anti-goat, or goat anti-mouse, dilution, 1:5000). Immunoreactive proteins were identified using the ECL Western blotting detection system (Santa Cruz Bio technology, CA, USA) and quantitated using a ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA).

### 4.8. Immunohistochemistry

Brain tissues fixed with 4% paraformaldehyde were transferred and incubated in a 30% sucrose solution in PBS for 48 h to ensure cryoprotection. Sectioned brain tissues (30 μm) using a sliding microscope adapted for cryosectioning were incubated overnight with anti-tyrosine hydroxylase (TH, Millipore, AB152, Germany) containing 10%...
normal donkey serum in PBS. Following washes (3×5 min), each tissue section was incubated with Horseradish peroxidase (HRP)-conjugated goat anti-rabbit for 2 h at room temperature, followed by avidin-biotin-peroxidase complex (1: 200; Vectastain-Elite ABC kit, Vector Laboratories, CA) for 30 min. After washing, the sections were reacted with diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CA, USA). Then, the tissue sections were dehydrated in 80%, 90%, 100% ethanol, became transparent using xylene and mounted using a mounting reagent, Permount. The slides were analyzed using the light microscope (Leica Microsystems, Wetzlar, Germany).

4.9. Immunofluorescence microscopy

Tissue sections were rinsed three times with PBS then, blocked with 10% normal donkey serum for 40 min. Primary antibodies for α-synuclein (BD biosciences, bd 610787, USA) and TLR2 (abcam, AB24192, Germany) were incubated overnight at 4 °C. Then the slides were washed (3×5 min) in PBS and pre-incubated with 2% normal donkey serum in PBS for 15 min, and transferred to a mixture of the secondary antibodies (cy3 conjugated donkey anti-mouse, 1:200 dilution, Jackson Immunochemicals, West Grove, PA, U.S.A. and TLR2, Alexa 488 conjugated donkey anti-rabbit, 1:200 dilution, Jackson Immuno chemicals, West Grove, PA, U.S.A.) for 2 h at room temperature. After several rinses, the sections were mounted on cover slides with Vectashield (Vector Laboratories, Burlingame, CA, USA). Tissue sections were examined using an immunofluorescence microscope (Leica Microsystems, Wetzlar, Germany). For negative controls, the primary antibodies were omitted.

4.10. Detection of apoptosis by TUNEL

TUNEL assay was used to detect in situ apoptotic cells with a commercial ApoTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA, USA). Briefly, the slides were incubated with 20 mg/ml proteinase K in PBS for 15 min followed by several washings in distilled water. After quenching with 3% H₂O₂ and applying the equilibrium buffer for 10 min, the sections were incubated in TdT enzyme for 1 h at 37 °C and cleaned with washing buffer for 10 min. The slides were rinsed in PBS (3×5 min), and then antidigoxigenin peroxidase conjugate was applied on the tissues for 30 min at room temperature. After washing in PBS, the peroxidase activity was visualized with DAB substrate kit (Vector Laboratories, Burlingame, CA, USA), and nuclei were counter-stained with 0.5% methyl green. The slides were examined using a fluorescent microscope (Leica Microsystems, Wetzlar, Germany).

4.11. Histological scoring of the apoptosis

Apoptotic cells, TUNEL positive cells in the striatum were identified when dark brown color is colocalized with nuclei. The number of apoptotic cells per 0.025 mm² was counted using an image analyzer (Analysis Pro 3.2, Sis Co., Munster, Germany). The resultant TUNEL positive cell numbers were considered as the total dead cell number per mm² in the striatum. Results were counted as mean ± SEM.

4.12. Statistical analysis

Data were analyzed using SPSS version 18.0 (SPSS, Chicago, IL, USA). All values are expressed as mean ± SEM. Statistical significance
was determined using an independent t-test or a one-way ANOVA when comparing the groups. A Bonferroni post hoc test was used for all pair-wise multiple comparisons when a statistically significant group main effect was found. Statistical significance was set at p < 0.05.

References


