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Fluoxetine prevents MPTP-induced loss of dopaminergic neurons by inhibiting microglial activation

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ABSTRACT

Parkinson's disease (PD) is characterized by degeneration of nigrostriatal dopaminergic (DA) neurons. Mice treated with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) exhibit microglial activation-induced oxidative stress and inflammation, and nigrostriatal DA neuronal damage, and thus serve as an experimental model of PD. Here, we report that fluoxetine, one of the most commonly prescribed antidepressants, prevents MPTP-induced degeneration of nigrostriatal DA neurons and increases striatal dopamine levels with the partial motor recovery. This was accompanied by inhibiting transient expression of proinflammatory cytokines and inducible nitric oxide synthase; and attenuating microglial NADPH oxidase activation, reactive oxygen species/reactive nitrogen species production, and consequent oxidative damage. Interestingly, fluoxetine was found to protect DA neuronal damage from 1-methyl-4-phenyl-pyridinium (MPP^+) neurotoxicity in co-cultures of mesencephalic neurons and microglia but not in neuron-enriched mesencephalic cultures devoid of microglia. The present in vivo and in vitro findings show that fluoxetine may possess anti-inflammatory properties and inhibit glial activation-mediated oxidative stress. Therefore, we carefully propose that neuroprotection of fluoxetine might be associated with its anti-inflammatory properties and could be employed as novel therapeutic agents for PD and other disorders associated with neuroinflammation and microglia-derived oxidative damage.

1. Introduction

Parkinson's disease (PD) is characterized by the progressive degeneration of nigrostriatal dopaminergic (DA) neurons and is associated with major clinical symptoms, including resting tremor, rigidity, and bradykinesia (Dauer and Przedborski, 2003; Savitt et al., 2006). The most prominent biochemical change in PD is a reduction in striatal dopamine levels that may result in a characteristic motor dysfunction. Although the etiology of PD is unknown, accumulating evidence suggests that PD is partly caused by glial activation, which may exert a neurotoxic effect through production of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) that, in turn, mediate oxidative stress (Gao et al., 2003; Liberatore et al., 1999; Wu et al., 2003). In the substantia nigra (SN) of PD patients and MPTP models of PD, key enzymes involved in the production ROS/RNS, such as microglial NAPDH oxidase and inducible nitric oxide synthase (iNOS), and astroglial myeloperoxidase (MPO), are upregulated in damaged areas and contribute to DA neuronal death (Choi et al., 2005a; Liberatore et al., 1999; Wu et al., 2003). In addition, proinflammatory cytokines, such as tumor

Abbreviations: DA neurons, dopaminergic neurons; PD, Parkinson's disease; SN, substantia nigra; STR, striatum; TH, tyrosine hydroxylase; iNOS, inducible NO synthase; ROS, reactive oxygen species; RNS, reactive nitrogen species; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; MDA, malondialdehyde.

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necrosis factor-α (TNF-α) and interleukine-1β (IL-1β), are also elevated and are involved in DA neuronal death in MPTP-treated mice (Zhao et al., 2007).

Fluoxetine, the selective serotonin reuptake inhibitor most commonly prescribed as an antidepressant, does not worsen parkinsonian motor symptomatology and is thus a safe and effective drug for treating depression in PD patients (Dell'Agnello et al., 2001). Recent studies revealed that fluoxetine inhibits the expression of the proinflammatory cytokines, TNF-α, IL-1β and cyclooxygenase-2 (COX-2), and suppresses nuclear factor κB (NF-κB) activity in the rat middle cerebral artery occlusion (MCAO) model of cerebral ischemia (Lim et al., 2009) and kainic acid (KA)-treated mouse hippocampus (Jin et al., 2009). However, little is known about the effects of fluoxetine, with respect to microglial activation, in the nigrostriatal DA system. Here, we show that in the MPTP mouse model of PD, fluoxetine prevents the degeneration of nigrostriatal DA neurons in a specific dose by inhibiting microglial activation and ultimately decreasing in ROS/RNS generation and oxidative stress.

2. Materials and methods

2.1. Animal and treatment

All experiments were done in accordance with approved animal protocol and guidelines established by Kyung Hee University. Eight-ten week old male C57BL6 mice (20–25 g, Charles River Breeding Laboratory) were used. For MPTP intoxication, mice received four intraperitoneal (i.p.) injection of MPTP (20 mg/kg free base; Sigma) dissolved in saline at 2 h interval. The fluoxetine treatment was done through injection of various doses (2.5 mg/kg body weight/single/day, 5 mg/kg/single/day, 5 mg/kg/twice/day, 10 mg/kg/single/day) into the peritoneum at the indicated time points, the first injection being 12 h after the last MPTP injection. Some mice were injected with fluoxetine alone or vehicle as a control. When calculated with mice body weight, 5 mg/kg/single/day fluoxetine is approximately equivalent to 0.1–0.125 mg/day.

2.2. Measurement of MPTP and MPP^+ levels in the striatum

Striatal MPP^+ level were measured by liquid chromatography electrosprayionisation mass spectrometry (LC/ESI-MASS). The LC/ESI-MASS system was composed of three Agilent model G1311A HPLC quaternary pumps (Palo Alto, CA, USA), a G1313A standard autosampler, and G1316A thermostatted column compartment. Dissected striatal tissues were sonicated and centrifuged at 9000 rpm, for 20 min in chilled 400 μl of 0.1 M perchloric acid, and 100 μl of supernatant was isocratically eluted through a 150 mm × 1.5 mm i.d., 4 μm, Zorbax Eclipse XDB-C18 (Palo Alto, CA, USA) maintained at 23 °C at flow rate of 0.2 ml min^{-1} for the separation of MPTP and MPP^+. The retention times of MPTP and MPP^+ were 6.967 and 7.763 min. An isocratic elution profile consist of 70% buffer A containing 0.1% formic acid in H_2O (v/v) and 30% buffer B containing 0.1% formic acid in acetonitrile (v/v). All samples were normalized for protein content, which was determined spectrophotometrically using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

2.3. Tissue preparation and immunostaining

Animals were transcardially perfused with a saline solution containing 0.5% sodium nitrate and heparin (10 U/ml) and then fixed with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB). Brains were dissected from the skull, postfixed overnight in buffered 4% paraformaldehyde at 4 °C, stored in a 30% sucrose solution at 4 °C until they sank, were frozen sectioned on a sliding microtome in 30-μm-thick coronal sections. All sections were collected in six separate series and processed for immunostaining as described previously (Kim et al., 2006). In brief, brain sections were rinsed in PBS and then incubated overnight at room temperature with primary antibodies. The following day, brain sections were rinsed with PBS and 0.5% bovine serum albumin (BSA), incubated with the appropriate biotinylated secondary antibody, and processed with an avidin–biotin complex kit (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). The bound antiserum was visualized by incubating with 0.05% diaminobenzidine-HCl (DAB) and 0.003% hydrogen peroxide in 0.1 M PB. The DAB reaction was stopped by rinsing tissues in 0.1 M PB. Labeled tissue sections were then mounted on gelatin-coated slides and analyzed under a bright-field microscope (Nikon, Mellville, NY). The primary antibodies were used anti-Mac-1 (1:200; Serotec, Oxford, UK) for microglia, anti-tyrosine hydroxylase (TH, 1:2000; Pel-Freez Biologicals, Rogers, AR, USA) for dopaminergic neurons, anti-neuronal nuclei (NeuN, 1:400; Chemicon, Temecula, CA), anti-CD68 (ED1, 1:1000; Serotec, Oxford, UK) for phagocytic microglia, anti-8-hydroxy-2′-deoxyguanosine (8-OHdG, 1:200; JaICA, Fukuroi, Shizuoka) for oxidative DNA, anti-nitrotyrosine (1:100; Abcam, Cambridge, UK). For Nissl staining, some of SN tissues were mounted on gelatin-coated slide and dried for 1 h at room temperature, stained with 0.5% cresyl violet (Sigma–Aldrich).

2.4. Stereological cell counts

The unbiased stereological estimation of the total number of the TH-positive cells in the substantia nigra (SN) was made using the optical fractionator (West et al., 1991), as we described in detail (Kim et al., 2005, 2006). The Computer-Assisted Stereological Toolbox system, version 2.1.4 (Olympus, Ballerup, Denmark) equipped with an Olympus BX51 microscope, a motorized microscope stage (Prior Scientific, Rockland, MA) run by an IBM-compatible computer, and a microcator (Heidenhain ND 281B) connected to the stage and feeding the computer with the distance information in the *z*-axis was used. The borders of the SN at all levels in the rostrocaudal axis were defined. The medial border was defined by a vertical line passing through the medial tip of the cerebral peduncle, by the medial terminal nucleus of the accessory nucleus of the optic tract for excluding the TH-positive cells in the VTA. The ventral border followed the dorsal border of the cerebral peduncle, including the TH-positive cells in the pars reticulata, and the area extended laterally to include the pars lateralis in addition to the pars compacta. The sections used for counting covered the entire SN from the rostral tip of the pars compacta back to the caudal end of the pars reticulate (anterioposterior, −2.06 to −4.16 mm from bregma) (Paxinos and Franklyn, 2001). The SN was delineated at a 1.25× objective and generated counting grid of 150×150 μm. An unbiased counting frame of known area ($47.87 \times 36.19\ \mu m = 1733\ \mu m^2$) superimposed on the image was placed randomly on the first counting area and systemically moved through all counting areas until the entire delineated area was sampled. Actual counting was performed using a 100× oil objective. The estimate of the total number of neurons was calculated according to the optical fractionator formula (West et al., 1991). More than total 300 points over all sections of each specimen were analyzed.

2.5. Densitometric analysis

As previously described (Ferger et al., 2004), an average of 17 coronal sections of the striatum, starting from the rostral anteroposterior (AP) (+1.60 mm) to AP (0.00 mm), according to bregma of the brain atlas (Paxinos and Franklyn, 2001), were examined at a 5× magnification using the IMAGE PRO PLUS system (Version 4.0, Media Cybernetics, Silver Spring, Maryland, USA) on a computer attached to a light microscope (Zeiss Axioskop, Oberkochen, Germany), interfaced with a CCD video camera (Kodak Mega Plus model 1.4 I, New York, NY, USA). To determine the density of the TH-immunoreactive staining in the striatum, a square frame of 700 × 700 μm was placed in the dorsal part of the striatum. A second square frame of 200 × 200 μm was placed in the region of the corpus callosum to measure background values. To control for variations in background illumination, the average of the background density readings from the corpus callosum was subtracted from the average of density readings of the striatum for each section. Then the average of all sections of each animal was calculated separately before data were statistically processed.

2.6. Rotarod test

To determine forelimb and hindlimb motor coordination and balance, we used an accelerating rotarod (UgoBasile, Comerio, Italy) with some modifications (Chung et al., 2010). The rotarod unit consisted of a rotating spindle (diameter, 3 cm) where mice were challenged for speed. To acclimate mice on the rotarod apparatus, animals were given a training session (10 rpm for 20 min), 7 consecutive days before MPTP injection. Animals that stayed on the rod without falling during training were selected and randomly divided into experimental groups. On 7 days from final MPTP injection, mice receiving various treatment regimes were placed on the rotating rod and tested at 20 rpm for 20 min. The latency to fall off the rotarod within this time period was recorded by magnetic trip plates.

2.7. Measurement of dopamine levels in the striatum

Levels of dopamine in striatum were measured by reverse-phase high performance liquid chromatography (HPLC) with electrochemical detector as previously described (Ryu et al., 2005). The isolated striata were homogenized and centrifuged at 9000 rpm for 20 min in 400 μl of 0.1 M perchloric acid and 0.1 mM EDTA. The 10 μl of supernatant was injected into an autosampler at 4 °C (Waters 717 plus autosampler) and eluted through μBondapak C18 column (3.9 × 300 mm × 10 μm, ESA) with mobile phase for catecholamine analysis (Chromosystems, Munich, Germany). The peaks of dopamine content were analyzed by ESA CoulochemII electrochemical detector and integrated using a commercially available program (Breeze, Waters Corp.). All samples were normalized for protein content as spectrophotometrically determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

2.8. Immunofluorescence double labeling

For double-immunofluorescence staining, tissue sections were processed as described previously (Park et al., 2009). Briefly, free-floating sections were mounted