

Original Article

## Major histocompatibility complex expression in a rotenone model of Parkinson's disease in rats

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**ABSTRACT** — Animal models can help determine the etiology of neurodegenerative diseases such as Parkinson's disease. Here, we conducted transcriptome analysis of the rotenone model of Parkinson's disease in rats. Exposure of 9-week-old Wistar rats to rotenone at 3 mg/kg/day for 14 days reduced spontaneous motor activity to 49% of that of control rats. Immunohistochemical analysis revealed increased expression of major histocompatibility complex (MHC) molecules in the substantia nigra of rotenone-treated rats, which was deduced by DNA array analysis. Further, gene set enrichment analysis of the transcriptome extracted the presence of a cytokine network, which included TNF- $\alpha$ . The expression of these proteins tended to be reduced at developed states of the disease. Thus, our analyses of the rotenone rat model still provides new insights into the etiology of Parkinson's disease.

**Key words:** Rotenone, Parkinson's disease, Transcriptome, Major histocompatibility complex, Cytokines

### INTRODUCTION

Environmental factors are considered to contribute to several types of sporadic diseases. In particular, juvenile dopaminergic neurons appear more vulnerable to environmental chemicals, such as endocrine-disrupting chemicals and pesticides (Jones and Miller, 2008; Elsworth *et al.*, 2013). A working hypothesis of the developmental origins of health and disease suggests that environmental origins early in life might induce development of dopaminergic neurodegenerative diseases late in life, such as Parkinson's disease (Gilman *et al.*, 2007). This phenomenon might be due to the early exposure to neurotoxic chemicals reducing the number of dopaminergic neurons in the substantia nigra below that required to sustain normal function during the course of neuronal attrition associated with aging. Indeed, we previously reported on structurally unrelated chemicals that cause developmental deficits in dopaminergic neurons in neonatal rats (Ishido *et al.*, 2002, 2004a, 2004b, 2004c and 2004d).

To test the hypothesis as a first step, we investigate a rat model of Parkinson's disease, which is a progressive neurodegenerative movement disorder that affects approximately 2% to 3% of the population in aged over 65 years (Parkinson, 1817; Mizuno *et al.*, 1998; Strange, 1992;

Kalia and Lang, 2015). In most Parkinson's patients, cardinal clinical symptoms include bradykinesia, resting tremor, rigidity, and postural instability. The major symptoms of Parkinson's disease result from the profound and selective loss of dopaminergic neurons in the substantia nigra pars compacta (Langston *et al.*, 1983). Parkinson's disease is primarily a sporadic disorder, and its etiology is not fully understood. An adult animal model of Parkinson's disease utilizing environmental compounds, such as rotenone, is therefore required (Betarbet *et al.*, 2000). Rotenone is derived from plants and commonly used as a pesticide (Ray, 1991), and chronic rotenone exposure reproduces a number of features of Parkinson's disease, including nigrostriatal dopaminergic lesions (Panov *et al.*, 2005; Inden *et al.*, 2007).

Here, to clarify the etiology of this disease, we investigated a rotenone rat model of Parkinson's disease.

### MATERIALS AND METHODS

#### Animals

Nine-week-old male Wistar rats were maintained in cages at 22°C with a 12-hr light-dark cycle on an MF diet (Oriental Yeast Corp., Tokyo, Japan) while receiving distilled water *ad libitum*. This study was conduct-

ed in strict accordance with the Experiment and Related Activities in Academic Research Institutions guidelines, under jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology. The experiment was approved by the Committee on the Ethics of Animal Experiments of the National Institute for Environmental Studies.

### **Subcutaneous implantation of osmotic pumps**

Subcutaneous implantation of osmotic pumps was conducted as previously described (Panov *et al.*, 2005). Alzet osmotic minipumps (Alzet Corp., Palo Alto, CA, USA) were filled with rotenone (Sigma-Aldrich, St. Louis, MO, USA), which was dissolved in equal volumes of DMSO and PEG 300 (Sigma). Pentobarbitone (50 mg/kg) was intraperitoneally injected for anesthesia. Alzet osmotic minipumps were implanted under the skin on the back of each rat. Control rats received DMSO:PEG (1:1). Rats received 3.0 mg of rotenone/kg/day (based on weight at time of surgery). Animals were sacrificed on Day 7 post-surgery.

### **Measurements of spontaneous motor activity**

Spontaneous motor activity was measured at 4-5 weeks of age, using a Supermex system (Muromachi Kikai, Tokyo, Japan), as described previously (Ishido *et al.*, 2002, 2004a). Briefly, a Supermex sensor head comprising paired infrared pyroelectric detectors was used to measure the radiated body heat of each animal. The system detected any object with a temperature at least 5°C higher than that of background within a cone-shaped area (6-m diameter; 110° vertex). Motion was monitored in several directions using an array of Fresnel lenses placed above the cage, so movement in the X, Y-, and Z-axes could be determined. Activity was measured at 15-min intervals for 22-24 hr under a 12-hr light-dark cycle. Food and water were fully given at the beginning of counting and the rats were not disturbed during the assessment period. Measurements from ten animals were recorded concurrently.

### **Transcriptome and gene set enrichment analyses**

Total RNA was extracted using an RNeasy Micro Kit (Qiagen GmbH, Hilden, Germany). Equivalent quantities of the total RNA obtained from three experiments were mixed for each analysis. Complementary DNA (cDNA) and biotin-labeled cRNA were synthesized using a One-cycle Target Labeling kit, and arrays were analyzed in accordance with the manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). Double-stranded cDNA was synthesized from 1 µg of total RNA and used

as a template for cRNA synthesis. Biotin-labeled cRNA (15 µg) was fragmented and hybridized to a GeneChip Rat Genome 230 2.0 Array. After hybridization for 15 hr, the array was washed, stained, and scanned. Data were analyzed using GeneChip Operating Software (GCOS ver. 1.4, Affymetrix). Array normalization with MAS 5.0 algorithm was applied to generate expression values using a scaling factor of 500.

DNA array data obtained were then processed with gene set enrichment analysis, using the Pathway Studio software (Ariadne Genomics, MD, USA).

### **Immunohistochemistry**

Immunostaining was conducted as previously described (Ishido *et al.*, 2004a, 2004b and 2004c). Rats were decapitated, and whole brain samples were fixed in 10% phosphate-buffered formalin (pH 7.2) and embedded in paraffin. Coronal sections were mounted on slides, deparaffinized in xylene, and hydrated through a series of graded ethanol. Sections were then permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS) and blocked with 10% normal horse serum plus 4% bovine serum albumin for 30 min at 4°C. Samples were incubated with antibodies against tyrosine hydroxylase (1:100; Sigma-Aldrich) or MHC I (1:50; Abcam, Tokyo, Japan) in the presence of 4% bovine serum albumin and 0.05% Triton X-100 overnight at 4°C. After three washes with PBS containing 0.1% Tween 20, the specimen with anti-tyrosine hydroxylase antibody was incubated with secondary antibody conjugated with FITC (1:200; Sigma-Aldrich) for 60 min at room temperature. Anti-MHC-I antibody was directly conjugated with FITC. Specimens were counterstained with 4',6-diamino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA, USA) for 5 min, as indicated and then observed using an Olympus IX-70 inverted fluorescence microscope (Olympus, Tokyo, Japan). Images were captured using the MetaMorph System (Molecular Devices, Downingtown, PA, USA) through a CoolSNAP ES monochrome digital camera (Nippon Roper, Tokyo, Japan).

### **Preparation of brain extracts and cytokine assay**

Whole brains were rapidly removed and put on an ice-cold glass plate with the ventral surface upwards in a cold box at 4°C. The midbrain was dissected, placed in a tube, frozen in liquid nitrogen, and stored at -80°C until use. Midbrain extracts were prepared using a Bio-Plex Cell Lysis kit (Bio-Rad, Hercules, CA, USA). Frozen tissue was thawed in cell lysis buffer, containing provided protease inhibitors and 5 mM phenylmethanesulfonyl fluoride (PMSF). Tissues were cut into 3 × 3 mm pieces and

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ground down in lysis buffer. After freezing and thawing, the samples were centrifuged at 4,500 g for 5 min. Supernatant was subjected to a cytokine assay of IL-2, IL-4, IL-6, IL-10, IL17, IL-18, and TNF- $\alpha$ . Protein concentrations were measured using a bicinchoninic acid kit (Pierce Chemical Co., Rockford, IL, USA) with bovine serum albumin as a standard.

### Statistical analysis

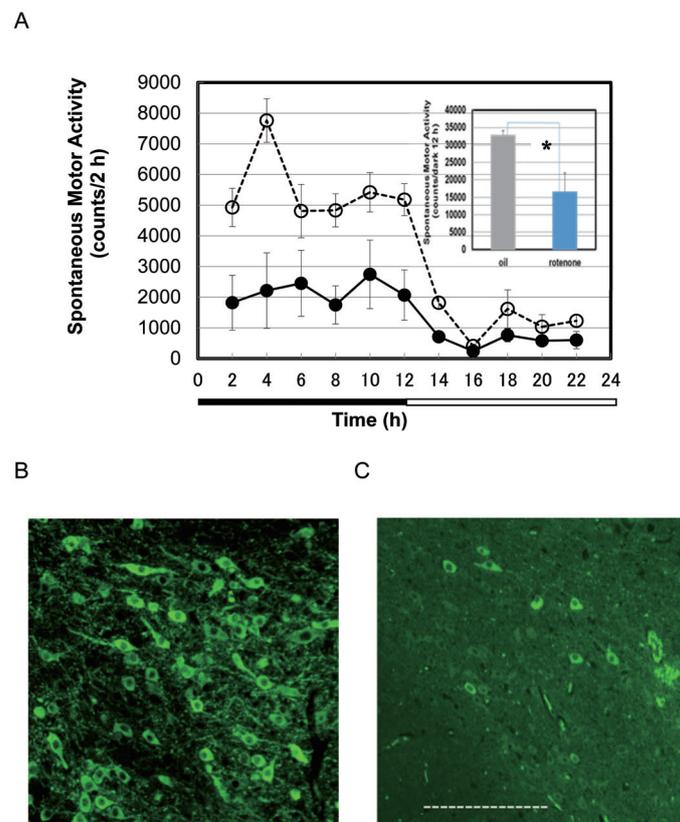
Statistical analysis was conducted by repeated measure ANOVA or Student's *t*-test using the StatView software package (ver. 6.0.3; SAS Institute Japan, Tokyo, Japan).  $P < 0.05$  was considered significant.

## RESULTS

Since motor impairment is a feature of Parkinson's dis-

ease, we focused our initial efforts on evaluating spontaneous motor activity (Supermex, CompACT AMS ver. 3.74, Muromachikikai Co., Tokyo, Japan). In the nocturnal phase, the rotenone-treated rats exhibited greater hypoactivity than vehicle-treated rats, as shown in Fig. 1A. In the diurnal phase, no significant differences in motor activity were detected between control and treated rats. The rhythmic pattern was similar between groups, indicating that the day-night cycle was retained. Hypoactivity was persistent in rotenone-treated rats during dark periods, with treated rats exhibiting an average of 49% less activity than vehicle-treated control rats (Fig. 1A, inset).

Immunohistochemical analysis was then conducted for tyrosine hydroxylase, which is a rate-limiting enzyme for catecholamine synthesis. Anti-tyrosine hydroxylase antibody provided strong staining in the substantia nigra of



**Fig. 1.** Characteristics of rat rotenone model of Parkinson's disease. (A) Spontaneous motor activity of rotenone-treated rats (filled circles) was measured using the Supermex system. Activity at 2-hr intervals is plotted. Control rats were administered vehicle alone (open circles). Data are indicated at mean  $\pm$  S.E. ( $n = 5$ ). Effects of rotenone on spontaneous motor activity were significant ( $p < 0.0001$  by ANOVA). Inset Spontaneous motor activity during nighttime was integrated. Data are shown as mean  $\pm$  S.E. \*Significantly different from control rats ( $p < 0.01$ , Student's *t*-test). (B) and (C) Immunohistochemistry for tyrosine hydroxylase. Brain sections of control (B) or rotenone-treated rats (C) were stained with anti-tyrosine hydroxylase antibody. Scale bar = 200  $\mu$ m.

control rats (Fig. 1B). In contrast, staining was markedly reduced in rotenone-treated rats, indicating degeneration of dopaminergic neurons (Fig. 1C).

To clarify the pathology of Parkinson's disease, a DNA array was conducted using a GeneChip Rat Genome 230 2.0 (Affymetrix). Total RNA was isolated from the mid-brain of 11-week-old rats and then transcribed to cDNA. Table 1 summarizes changes in gene expression of major molecules in the midbrain. Notably, MHC molecules exhibited the largest increase in response to rotenone. To confirm this finding regarding MHC, we immunostained the brain tissues of 11-week-old rats with the anti-MHC class I antibody OX-18. In rotenone-treated rats, OX-18-positive proteins were observed in substantia nigra, with a markedly lower number being observed in control tissues (Fig. 2).

Transcriptome data by DNA array underwent subsequent gene set enrichment analysis. Fig. 3 shows the extraction from the transcriptome, with the emergent clusters indicating a cytokine network that includes TNF- $\alpha$ .

We quantitatively measured the concentration of cytokines, which were predicted by gene set enrichment analysis to be involved in neurodegeneration. Mid-brain tissues of 11-week-old rats were homogenized and then subjected to a Bio-Plex system (Bio-Rad, Hercules, CA, USA). Fig. 4 shows that rotenone exposure tended to decrease the concentration of all cytokines tested by approximately 10%-20%, with the significant exception of TNF- $\alpha$  at 45% ( $p < 0.05$ ).

## DISCUSSION

In this study, we demonstrate the neuronal MHC-I expression in the rotenone rat model of Parkinson's disease with low concentration of proinflammatory cytokines such as IL-6, IL-17, IL-18, and TNF- $\alpha$  as well as anti-inflammatory cytokines such as IL-4 and IL-10 in the mid-brain.

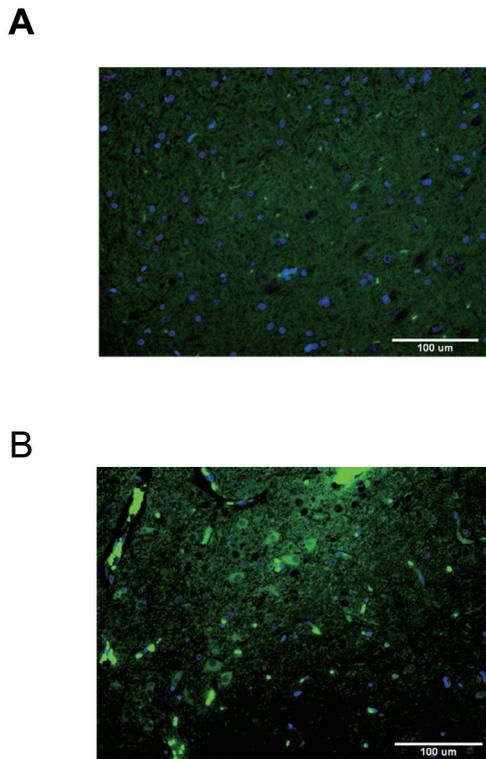
To better understand the etiology of human Parkinson's disease, a number of animal models have been developed, including genetic and neurotoxic models (Dauer and Przedborski, 2003; Blesa *et al.*, 2012; Segura-Aguilar *et al.*, 2015; Taylor *et al.*, 2010; Cannon and Greenamyre, 2013). Both genetic and environmental factors likely contribute to the prevalence of sporadic Parkinson's disease. Environmental factors appear to include drinking well water, rural living, beta-blocker use, agricultural occupation, and pesticide exposure (Kalia and Lang, 2015). Of these models, the rotenone model is the most environmentally relevant (Cannon and Greenamyre, 2013).

Gene expression profiling is a basic approach to reveal the molecular etiology, and a number of reports have been published regarding DNA array data obtained from post-mortem brain samples of Parkinson's disease patients and mammalian models (Green, 2012). However, data from postmortem samples of Parkinson's disease patients are affected by issues of sample size, sample composition, and disease stage, even when using laser-capture microdissection (Green, 2012). Despite these issues, Zheng *et al.* (2010) reported genome-wide meta-analyses in samples from patients with symptomatic Parkinson's disease

**Table 1.** Gene expression in the midbrain of rotenone model of Parkinson's disease.

Gene ID	Gene name	Expression ratio (log2 ratio)
100529072	RT1 class Ib, Ia locus Aw2	3.7
6817	Sulfotransferase family 1A	2.9
3158	HMG-CoA synthase	2.4
1891701	Peroxisomal membrane protein 4	2.3
60666	Glycerol-3-phosphate dehydrogenase 1	2.2
29517	Glucocorticoid regulated kinase	2.2
307799	Par-6 partitioning defective 6 homolog	1.8
219935278	Lipocalin 7	1.7
24653	Phospholipase A2	1.7
114851	Cyclin-dependent kinase inhibitor	1.6
64032	Connective tissue growth factor	1.6
361619	Beta-glo	-2.2
25632	Hemoglobin	-2.1
65155	Aminolevulinic acid synthase	-1.7
24203	Amylase 1	-1.6
116640	Tenascin C	-1.5
24232	Complement component 3	-1.5

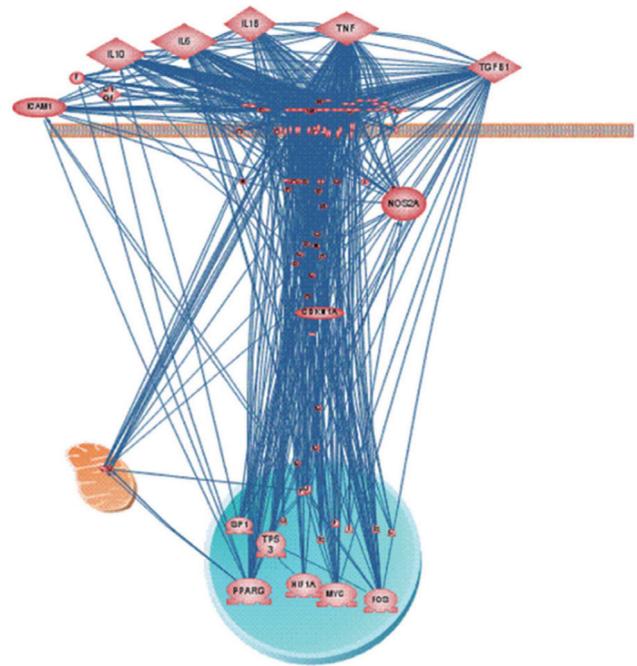
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**Fig. 2.** OX18 immunohistochemistry in substantia nigra. After exposure of adult rats to vehicle (A) or rotenone (B), coronal sections of brain tissues were stained with anti-OX-18 antibody, followed by counterstaining with DAPI, as described in Section 2.4. Scale bar = 100 µm.

and subclinical disease and healthy controls, and that all gene sets were related to bioenergetics. Our gene set analysis suggests that connective tissue growth factor might be a mediator of events driven by TGF- $\beta$ 1 (Fig. 3), which is consistent with a previous finding (Grotendorst, 1997). Lipocalin 7 was also reported to be sensitive to TGF $\beta$ 1 (Brown, 2010). Notably, Richter *et al.* (2009) reported that gene expression of hemoglobin was down-regulated by the mitochondrial inhibitor rotenone, which is consistent with our present data. Our transcriptome analysis also revealed that other mitochondrial proteins such as HMG-CoA synthase and glycerol-3-phosphate dehydrogenase 1 appear to be affected by rotenone exposure (Table 1).

One of the advantages of animal models over human samples is the ability to examine animals that are asymptomatic or in the early stage of the disease. In this regard, Meurers *et al.* (2009) reported the changes in apoptosis-related transcripts in rotenone-treated rats with mild impairment of motor function. Further, Greene *et al.* (2010) reported the alteration of metabolism-related transcripts in rotenone-treated rats with normal behavior. In

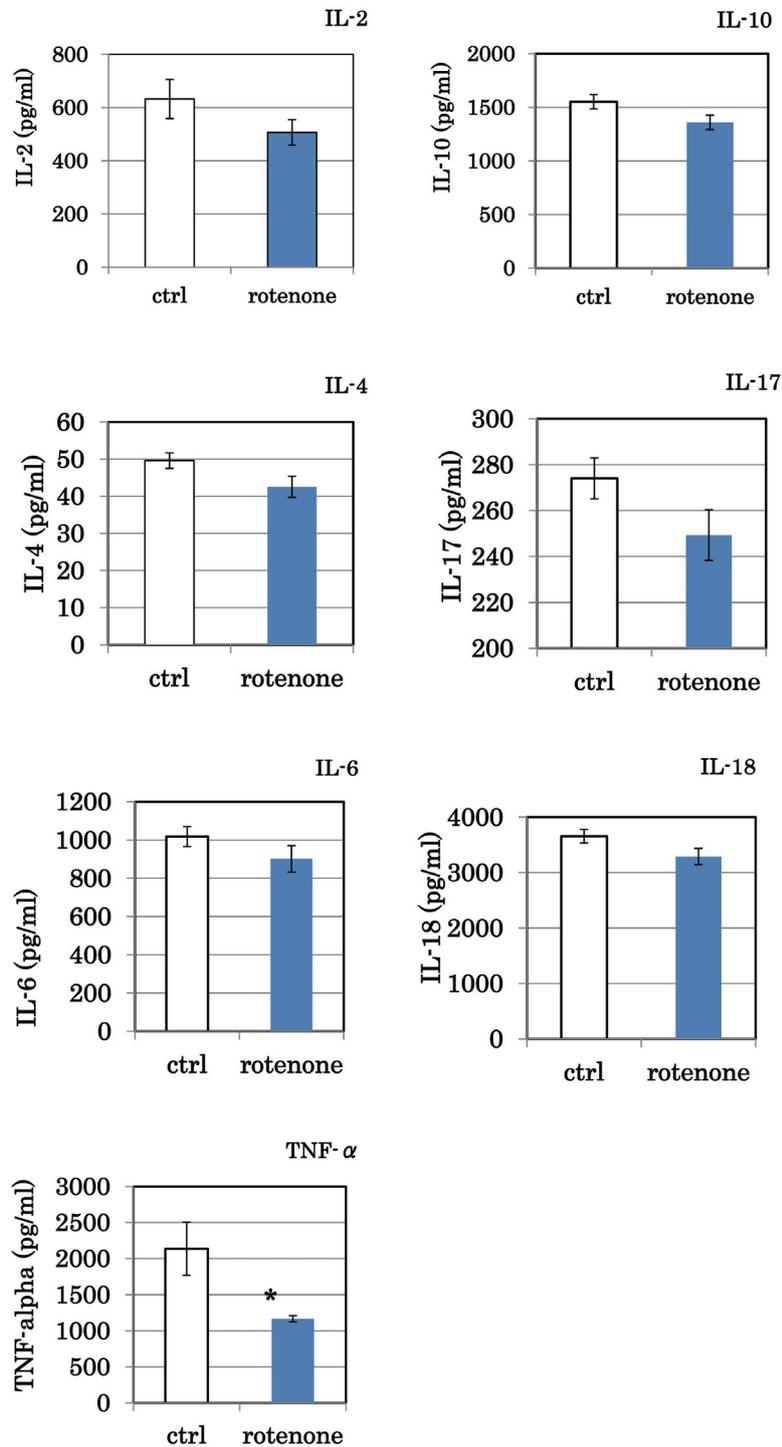


**Fig. 3.** Schematic representation of gene network deduced from transcriptome of midbrain of rotenone-treated rats. DNA array data were analyzed using Pathway Studio software.

the present study, however, we used rotenone-treated rats in the advanced stages of disease, observing a 49% decrease in spontaneous motor activity compared to activity in untreated rats (Fig. 1). Further, we subjected whole midbrains to DNA array analyses, which identified MHC genes as the most sensitive to rotenone (Table 1). We also conducted a gene set enrichment analysis of the transcriptome, which predicted the cytokine network (Fig. 3).

Presence of MHC in neuron was demonstrated by Neumann *et al.* as well as in glial cells (Wong *et al.*, 1984). Localization of MHC mRNA in various brain regions suggests to be involved in the early neurodevelopment (Linda *et al.*, 1999; Huh *et al.*, 2000). Neural MHC expression is considered to play a role in early developmental synaptic plasticity (Cebrián *et al.*, 2014a, 2014b; Elmer and McAllister, 2012; Shatz, 2009). Further, Cebrián *et al.* (2014a, 2014b) demonstrated that MHC I molecules are expressed by substantia nigra dopaminergic and locus coeruleus neurons in human postmortem samples of adult controls and Parkinson's disease patients. Notably, Na *et al.* (2010) also observed upregulation of MHC gene expression in a 6-hydroxydopamine model of Parkinson's disease.

Several neurodegenerative disorders, including Parkinson's disease, involve components of neuroinflammation (Tansey and Goldberg, 2010). The concentration of cytokines such as IL-2, IL-4, IL-6, IL-10, TNF- $\alpha$ ,



**Fig. 4.** Quantification of cytokines in midbrain of rotenone-treated rats. Nine-week-old male Wistar rats were treated with vehicle alone (ctrl) or 3 mg/kg/day of rotenone (rotenone) for 14 days, as indicated. Brain tissue samples at 11 weeks were homogenized on ice in lysing buffer with proteinase inhibitors. After freezing and thawing, homogenates were centrifuged at 4,500 g for 4 min. Supernatants were then subjected to a Bio-Plex system. Assay was conducted in triplicate and data are represented as mean  $\pm$  S.E. (n = 5). \*p < 0.05 considered significant.

and IFN- $\gamma$  increased in the serum of Parkinson's disease patients (Brodacki *et al.*, 2008; Reale *et al.*, 2009). IL-10 and IL-17 gene polymorphism (Xu *et al.*, 2010) and IL-18 promoter polymorphism (Nie *et al.*, 2013) are reportedly associated with risk of developing sporadic Parkinson's disease. These observations suggest that an active inflammatory process might occur in the brain of Parkinson's disease patients.

In contrast, cytokine concentrations tended to be low in our model—statistically significantly so for TNF- $\alpha$ , a key pro-inflammatory cytokines (Fig. 4). This suggests that microglia were resting state, while MHC was activated (Fig. 2B) and dopaminergic neurons were lost (Fig. 1C) at the time examined. Rotenone does not directly activate microglia or induce cytokines (Klintworth *et al.*, 2009). Dynamic changes of cytokines levels were seen in several neurodegeneration (Klusman and Schwab, 1997; Moghaddam *et al.*, 2015). It is possible that feedback mechanisms might inhibit the secretion of these cytokines, as the models examined were in the severe stage of the disease. TNF- $\alpha$  exhibits both neurotoxic as well as neuroprotective actions after brain injury. These dual roles of TNF- $\alpha$  have been demonstrated in animal models of Parkinson's disease (Chertoff *et al.*, 2011; Montgomery and Bowers, 2012; Sadek *et al.*, 2014)

Furthermore, it has been reported that MHC-I expression renders catecholaminergic neurons susceptible to T-cell-mediated degeneration (Tansey and Goldberg, 2010). They demonstrated that dopaminergic neurons internalize foreign ovalbumin and display antigen derived from this protein by MHC-I, which triggers dopamine neuronal death in the presence of appropriate cytotoxic T cells. Thus, future study will be required to clarify the kinetics of cytokines in parallel with those of neural MHC activation.

Although the rotenone model of Parkinson's disease has been well investigated, there are no reports of neural MHC activation in this model. Our findings might help to clarify the pathological mechanism behind the progression of neural loss. The model still hides the riches to be investigated.

## ACKNOWLEDGMENTS

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**Conflict of interest----** The authors declare that there is no conflict of interest.

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