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Low-grade inflammation aggravates rotenone neurotoxicity and disrupts circadian clock gene expression in rats

Huan Li^{1,3}, Sheng Song², Yuan Wang¹, Chun Huang¹, Feng Zhang¹, Jie Liu^{1,2}, and Jau-Shyong Hong²

¹Key Laboratory of Basic Pharmacology of Ministry of Education and Joint International Research Laboratory of Ethnomedicine of Ministry of Education, Zunyi Medical University, Zunyi, Guizhou, China

²National Institute of Environmental Health Sciences, NIH, Research Triangle Park, NC 27709, USA

³Jiangsu Key Laboratory of Drug Discovery for Metabolic Disease, China Pharmaceutical University, Nanjing, China, 210023

Abstract

A single injection of LPS produced low-grade neuroinflammation leading to Parkinson's disease (PD) in mice several months later. Whether such a phenomenon occurs in rats and whether such low-grade neuroinflammation would aggravate rotenone (ROT) neurotoxicity and disrupts circadian clock gene/protein expressions were examined in this study. Male rats were given two injections of LPS (2.5–7.5 mg/kg), and neuroinflammation and dopamine neuron loss were evident 3 months later. Seven months after a single LPS (5 mg/kg) injection, rats received low doses of ROT (0.5 mg/kg, sc, 5 times/week for 4 weeks) to examine low-grade neuroinflammation on ROT toxicity. LPS plus ROT produced more pronounced non-motor and motor dysfunctions than LPS or ROT alone in behavioral tests, and decreased mitochondrial complex 1 activity, together with aggravated neuroinflammation and neuron loss. The expressions of clock core genes brain and muscle Arnt-like protein-1 (Bmal1), locomotor output cycles kaput (Clock), and neuronal PAS domain protein-2 (Npas2) were decreased in LPS, ROT and LPS plus ROT groups. The expressions of circadian feedback genes Periods (Per1 and Per2) were also decreased, but Cryptochromes (Cry1 and Cry2) were unaltered. The circadian clock target genes nuclear receptor Rev-Erba (Nr1d1), and D-box-binding protein (Dbp) expressions were also decreased. Consistent with the transcript levels, circadian clock protein BMAL1, CLOCK, NR1D1 and DBP were also decreased. Thus, LPS-induced chronic low-grade neuroinflammation potentiated ROT

Corresponding authors: Jie Liu, Jie@liuonline.com;.

AUTHOR CONTRIBUTIONS

Huan Li, Jie Liu, Sheng Song, Feng Zhang and John Hong conceived and designed the experiments. Huan Li, Jie Liu, Sheng Song, Yuan Wang, Chun Huang participated in the experiments performance and data analysis. Huan Li, Sheng Song, Jie Liu, Feng Zhang and John Hong wrote and revised the article. All the authors have reviewed the contents of the manuscript and approved the submitted manuscript.

Conflict of Interest Statement:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

neurotoxicity and disrupted circadian clock gene/protein expression, suggesting a role of disrupted circadian in PD development and progression.

Keywords

Parkinson's disease; lipopolysaccharide; rotenone; non-motor dysfunction; neurotoxicity; circadian clock gene expression

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder in elderly (Pringsheim et al. 2014). Accumulating evidence indicates a close association between inflammatory responses and PD (Qin et al. 2007; Stojkovska et al. 2015). Neuroinflammation is a self-defense reaction to combat pathogen infection and typically transient and help maintain brain homeostasis. However, neuroinflammation might continue unresolved becoming persistent in pathological conditions (Block et al. 2007; Chen et al. 2016). For example, a single intraperitoneal injection of bacterial lipopolysaccharide (LPS) elicited a low-grade, long-lasting neuroinflammation leading to dopaminergic neuron loss and PD pathology several months later in mice (Qin et al. 2007, 2013; Gao et al. 2003, 2008).

Rotenone (ROT), a pesticide, has been used to produce PD animal models (Johnson and Bobrovskaya 2015). Evidence suggests that ROT facilitates DA neuron loss at least in part by induction of inflammation (Ramsey and Tansey 2012). Previous study showed that LPS and ROT interact with each other to induce toxicity in primary mesencephalic cultures (Gao et al. 2003). Intraperitoneal injection of LPS in neonate rats enhances susceptibility of nigrostriatal dopaminergic neurons to ROT neurotoxicity later in life (Cai et al. 2013). We have recently demonstrated that a single injection of LPS potentiated neurotoxicity of ROT in rats, leading to microglia activation, dopamine neuron loss in substantia nigra and the decreased levels of dopamine and its metabolites in striatum (Huang et al. 2017).

It's has been well acknowledged that PD impairs both motor and non-motor functions (Sjödahl et al. 2012; Schapira et al. 2017). Most of the motor symptoms of PD could be preceded, sometimes for several years, by non-motor symptoms that include hyposomnia, sleep disorders, depression and constipation (Schapira et al. 2017). Circadian clock dysregulation is one of the important but ignored non-motor symptoms in PD (Willison et al. 2013; Videnovic et al. 2014). Circadian dysfunction has been observed in PD patients and in animal PD models including mice, rats, dogs, and non-human primates (Videnovic and Willis 2016), which may result in negative consequences to the homeostasis and even exacerbate the disease progression (Chahine et al. 2016; Li S et al. 2017; Willison et al. 2013). Circadian rhythm dysfunction is now considered as a novel environmental risk factor for PD (Lauretti et al. 2016), and a novel diagnostic and therapeutic target in PD and neurodegenerative disorders (Videnovic and Willis 2017).

Circadian clock, an endogenous timing system, is located in every cell and organ, which was regulated and maintained by transcriptional-translational feedback loops (Buhr and Takahashi 2013; Partch et al. 2014). At the core of this clock network are the transcriptional

activators Clock, Bmal1, and Npas2, which heterodimerize CLOCK/BMAL1 and NPAS2/BMAL1 and bind to E-box, driving transcription. Bmal1/CLOCK drives transcription of several distinct negative feedback genes, including two Cryptochrome (Cry1, Cry2) genes and three Period genes (Per1, Per2, Per3). Per and Cry proteins then heterodimerize and repress Bmal1/Clock-mediated transcription and repress their own expression. The CLOCK-BMAL1 heterodimer also governs the robustly circadian expression of nuclear orphan receptors Rev-Erba (Nr1d1) and the PAR-bZip family members Dbp (Mohawk et al. 2012; Partch et al. 2014). Aberrant expression of circadian clock genes could result in dysregulated circadian rhythm at earlier stages of PD and the progression of the disease (Videnovic and Willis 2016; Schapira et al. 2017).

Circadian disruption has been reported to dysregulate inflammatory responses (Castanon-Cervantes et al. 2011), impair tissue homeostasis and exacerbate chronic inflammation, such as in the intestine (Pagel et al. 2017). However, little is known about circadian disruption in chronic LPS-induced neuroinflammation.

We have shown that chronic LPS exacerbated ROT-induced dopaminergic neuronal damage in adulthood rat model (Huang et al. 2017). Since the brain samples were collected in the morning from 9:00 to 11:00 am, we motivated to further examine the expression of circadian clock core genes, feedback control genes, and circadian clock-driven genes at the transcription and translation levels in this LPS combined with ROT-induced neurodegeneration in rats.

2. Materials and methods

2.1 Reagents

Lipopolysaccharide (LPS, 0111:B4) and rotenone (ROT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Polyclonal mouse anti-Tyrosine Hydroxylase (TH) antibody (MAB318) was from MABEMD Milipore (Temecula, CA); Polyclonal rabbit anti Iba-1 antibody (019–19741) was from Wako (Richmond, VA); Antibodies against BMAL1 (ab93806), CLOCK (ab134165) were from Abcam (Cambridge, MA), antibodies against NR1D1 (ARP33733_P050) and DBP (ARP32195_P050) were from AVIVA Systems Biology (San Diego, CA). β -actin (Catalog No. mAb 4970, rabbit monoclonal) was purchased from Cell Signaling Technology (Beverly, MA). Mitochondrial Complex 1 kit was from Cominbio (Suzhou, China).

2.2. Animals and Treatment

Male Sprague-Dawley rats were purchased from the Experimental Animal Center of the Third Military Medical University (Chongqing, China; Certificate No. SCXK 2012–0011), and were fed rodent chow in the SPF-grade animal facilities with $21 \pm 2^\circ\text{C}$ and the light on from 8:00 to 20:00 at Zunyi Medical College. All animal experiments were performed in accordance with Chinese Guidelines of Animal Care and Welfare and the present study was approved by the Animal Care and Use Committee of Zunyi Medical University (2014–07).

To affirm LPS-induced neuroinflammation in rats, adult rats (220 g) were given two injections of LPS (2.5, 5.0, and 7.5 mg/kg, $n=3$) in continuously two days, and 3 months

later, brains were collected, fixed in 4% paraformaldehyde to examine microglia activation via Iba1 immunostaining and dopamine neuron loss via tyrosine hydroxylase (TH) immunostaining (detailed below).

Based on this preliminary study, a single intraperitoneal injection of LPS (5 mg/kg) was used to produce low-grade neuroinflammation. Seven months later, rats received subcutaneous injection of low dose of ROT (0.5 mg/kg) or corn oil (an equivalent volume of ROT) five times a week for consecutive 4 weeks (Ojha et al., 2015; Huang et al., 2017). After the last treatment of ROT, rats were allowed to recover for an additional 10 days before behavior tests. At the 14th day after the last ROT treatment, brain tissues were collected (Control = 9, LPS = 7, ROT = 6, LPS+ROT = 8). To minimize the potential time-differences, all samples were collected within two hours (9:00–11:00) and one animal for each group were killed in turn to minimize the time-difference in sample collection among groups. Samples were stored –80°C prior to analysis (Huang et al. 2017).

2.3. Non-motor behavioral tests

All behavioral data were captured by a video-monitor and analyzed using TopScan Lite 2.0 (CleverSys, Reston, VA, USA). The open-field test was used for non-motor behavioral activity (Bergamini et al. 2017). The elevated plus maze test is one of the most widely used tests for measuring anxiety-like behavior. The test is based on the natural aversion of animals for open and elevated areas, as well as on their natural spontaneous exploratory behavior in novel environments. The apparatus consists of open arms and closed arms, crossed in the middle perpendicularly to each other, and a center area (Komada et al. 2008). Y maze test was performed for learning memory. Successful alternation behavior was defined as entry into all three arms consecutively. The percentage of successful alternation was calculated as the ratio of the alternative to non-alternatives (Jin et al. 2014).

2.4. The activity of mitochondria complex 1

Mitochondrial complex1, also known NADH-CoQ reductase, is the largest protein complex in the inner membrane of mitochondria. Chronic systemic administration of rotenone, a specific complex 1 inhibitor, resulted in neuropathologic and behavioral changes in rats that are similar to human PD (Luo et al. 2015). Thus, the activity of mitochondria complex1 in the brain was examined using the mitochondria complex1 assay kit (Cominbio, Suzhou, China), and results were expressed as the percentage of controls.

2.5. Immunohistochemistry

Rat brains were cut into 35 µm sections on a horizontal sliding microtome. The free-floating sections were immunoblocked with 4% goat serum in 0.25% triton/PBS for 2 hours and then incubated with Iba-1 (1:2000), antibody overnight at 4 °C. On the second day, the sections were washed by 1% BSA in 0.25% triton/PBS before the incubation with anti-TH antibody (1:2000) overnight at 4 °C. The double-label immunofluorescence pictures were taken under the confocal microscope by using Alexa-488 (green) and Alexa-594 (red) conjugated secondary antibodies (1:1,000) to visualize the THir or Iba-1-positive cells (Jiang et al., 2015). The number of THir and Iba-1+ cells was counted as described previously in our Lab (Liu et al., 2008). Briefly, eight evenly spaced brain slices from saline or LPS-injected rats

were immunostained with an antibody against TH or Iba-1 and counted. The distribution of the cell numbers from each rat was matched with the normal distribution curve to correct for errors resulting from the cutting. Samples were counted in a double-blind manner with stereology equipment. Data were expressed as percent loss compared to saline-injected controls.

2.6. Total RNA isolation and RT-qPCR analysis

Total RNA was extracted with TRIzol (TakaRa Biotechnology, Dalian, China). The quality and quantity of RNA were determined by Nano Drop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA), with 260/280 > 1.8, and by gel electrophoresis. Total RNA was reverse transcribed to cDNA with TakaRa RT kits (Dalian, China) according to manufacture instructions. The 15 µl PCR reaction mix contained 3 µl of cDNA (10 ng/ml), 7.5 µl of iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 0.5 µl of primer mix (10 mM each), and 4 µl of ddH₂O. After 5 min denature at 95 °C, 40 cycles were performed: annealing and extension at 60°C for 45 s and denature at 95°C for 10s in CFX96 Touch™ Real-time PCR system (Bio-Rad Laboratories, Hercules, CA). Dissociation curve was performed after finishing 40 cycles to verify the quality of primers and amplification. The Ct values were used to calculate the expression of clock genes by the $2^{-\Delta\Delta C_t}$ method and normalized to the housekeeping gene β -actin and GAPDH. Both β -actin and GAPDH yielded similar results, and thus the expression of interested genes was first normalized with β -actin of the same sample, and the relative transcript levels were calculated setting control as 100% as described (Li et al., 2017a; 2017b). The primers were designed with Primer3 software and listed in Table 1.

2.7. Western Blotting Analysis

Approximately 50 mg cortex tissues were homogenized in 1 mL RIPA lysis buffer containing protease inhibitors (100 µM phenyl methyl sulfonyl fluoride) using an electric homogenizer. Supernatant were prepared by centrifugation at 14 000 g for 15 min. Protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime, China). Protein (30 µg) were separated on 10% Bis-Tris Nu-PAGE gel by electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk and incubated over night at 4°C with the following primary antibodies: anti- β -actin (1:5000), BMAL1 (1:1000), CLOCK (1:10000), NR1D1 (1:1000), DBP (1:1000). After thorough wash in TBST, the blots were incubated with horseradish peroxidase- conjugated secondary antibodies at 1:1000 dilutions for 2 h. Immunoblots were visualized using chemiluminescence reagent BeyoECL Combined with (Beyotime, Shanghai, China). The image was scanned and optical density values were quantified using Adobe Photoshop CS5 software (Huang et al. 2017).

2.8. Statistical analysis

All data were expressed as mean $\pm$ SEM. Comparison of multiple groups was performed using one-way ANOVA analysis followed by Social Sciences (SPSS) software (version 18.0), and $p < 0.05$ was considered significantly different.

3. Results

3.1. LPS induced chronic neuroinflammation and dopaminergic neuron loss in substantia nigra

Loss of dopaminergic neurons in the substantia nigra is a main character in PD patients and PD animal models (Pringsheim et al. 2014), which is associated with neuroinflammation in the area (Block et al. 2007; Chen et al. 2016). A single injection of LPS produced chronic neuroinflammation in mice (Qin et al. 2007, 2013), whether injection of LPS could also produce neuroinflammation in rats is not known. A study was therefore conducted to affirm the LPS-induced neuroinflammation in rats with three different doses. As illustrated in Figure 1, three months after injections of LPS (2.5, 5.0, and 7.5 mg/kg) in two consecutive days, LPS induced microglia activation in a dose-dependent manner, characterized by hypertrophied morphology and intensified Iba1 staining (green). The photo of the enlarged, ramified microglia was shown in Supplemental Figure 1. Meanwhile, the numbers of microglia were increased by 6%, 25% and 35% after LPS 2.5, 5.0 and 7.5 mg/kg, respectively. Upon microglia activation, dopamine neurons loss was evident, as evidenced by reduced THir neurons (red). The numbers of THir cells were decreased by 20%, 43% and 55% after LPS 2.5, 5.0 and 7.5 mg/kg, respectively. A whole brain image of microglia activation and THir neuron loss is shown in Supplemental Figure 2. These results demonstrated that LPS injections induced chronic neuroinflammation in rats causing delayed loss of THir neurons in substantia nigra, a brain region sensitive to LPS (Qin et al. 2007, Huang et al. 2017). However, in this study, 30% rats in the 7.5 mg/kg group died, and we have thus used 5 mg/kg LPS, the same dose as used in mice (Qin et al. 2007, 2013; Liu et al. 2008), but for longer-time periods.

3.2. LPS exacerbated ROT produced non-motor behavioral changes

Chronic LPS-induced low-grade neuroinflammation potentiated ROT on non-motor alternations. Fig. 2A and 2B showed the results from the open-field tests. Rats travelled (10 min) 2780 ± 390 cm in control group, 2660 ± 390 cm in LPS group, and 1670 ± 360 cm in rotenone (ROT) group, and further decreased to 1280 ± 320 cm in LPS + ROT group (Fig. 2A). The exploratory activity was also reduced in LPS + ROT group, and rats were mainly remained in local area (Fig. 2B). In the elevated combined with maze test, ROT and LPS + ROT groups showed the tendency of reduced activity in open arms (Fig. 2C). In the Y-maze teste, the correct spontaneous alternatives, a test for learning and memory, was also tended to decrease in LPS + ROT group. The values of three groups were 69%, 64%, 61%, and 33% in LPS + ROT group, the percentage of spontaneous alteration was decreased from 69% to 33% (Fig. 2D), indicating LPS + ROT could interfere with the learning and memory function, despite it was not statistically significant. Taken together with Rotarod test for motor activities (Huang et al. 2017), LPS aggravated ROT in producing behavioral deficits.

3.3. LPS combined with ROT decreased mitochondrial function

ROT is a well-known neurotoxicant damaging mitochondria, especially mitochondrial complex 1 (Sharma et al. 2013; Johnson and Bobrovskaya 2015). Thus, mitochondrial function was examined. As shown in Figure 3, compared with the control group, the activity

of mitochondria complex1 was decreased 29% in ROT group but 46% in LPS plus ROT group.

3.4. LPS combined with ROT decreased the expression of circadian clock core genes and proteins

Circadian clock core genes include Bmal1, Clock, Npas2 (Buhr and Takahashi 2013; Partch et al. 2014). Compared with the control group, the mRNA expression of Bmal1 decreased 11.3% in LPS group, 24.9% in ROT group, and 32.1% in LPS plus ROT group (Fig. 4A). Consistent with the mRNA expression of Bmal1 gene, the expression of BMAL1 protein was also decreased in LPS, ROT and LPS plus ROT group (the decrease was up to 29.8%) (Fig. 4B). For mRNA expression of Clock gene, compared with the control group, its expression decreased 18.5% in LPS group, 23.1% in ROT group and 32.8% in LPS plus ROT group (Fig. 4A). Compared with the control group, CLOCK protein decreased 32.3% in ROT group and 39.1% in LPS plus ROT group (Fig. 4C). Expression of Npas2 mRNA decreased 25.5%, 32.7% and 42.6% in LPS, ROT and LPS plus ROT groups, respectively. Due to the individual variation, only in LPS plus ROT group, the decrease of Npas2 was significant (Fig. 4A).

3.5. Effects of LPS and ROT on the expression of clock feedback genes

Circadian clock feedback genes include Per1, Per2, Cry1, and Cry2 (Buhr and Takahashi 2013; Partch et al. 2014). For Per1 gene, its expression decreased in LPS, ROT and LPS plus ROT group. The decrease was 25.6% in LPS plus ROT group that was significant. For Per2 gene, compared with the control group, Per2 expression decreased 28.7% in LPS group, 45.1% in ROT group and 57.8% in LPS plus ROT group, respectively. As shown in Figure 5, compared with the control group, mRNA expressions of Cry and Cry2 had no significant changes in LPS, ROT and LPS plus ROT groups.

3.6. LPS combined with ROT decreased the expression of clock targeted genes and proteins

Circadian clock targeted genes include Nr1d1 and Dbp (Buhr and Takahashi 2013; Partch et al. 2014). As shown in Figure 6, expressions of Nr1d1 and Dbp were decreased not only in mRNA but also in protein levels. For Nr1d1 gene, compared with the control group, its expression decreased 13.3% in LPS group, 16.6% in ROT group, and 22.4% in LPS plus ROT group (Fig. 6A). At the protein level, the expression of NR1D1 decreased in LPS, ROT and LPS plus ROT groups. Especially in LPS plus ROT group, the decrease was up to 25.9% that was significant (Fig. 6B). For Dbp gene expression, compared with the control group, its expression decreased 13.8% in LPS group, 19.0% in ROT group and 29.5% in LPS plus ROT group (Fig. 6A). At the protein level, the expression of DBP decreased in LPS, ROT and LPS plus ROT groups. Compared with the control group, DBP decreased 28.1% in LPS plus ROT group that was significant (Fig. 6C).

4. Discussion

The present study clearly demonstrated that approximately 3 months after LPS injection in rats, neuroinflammation and neuron loss were evident, duplicates that observed in mice (Qin

et al. 2007). In our prior studies, a single systemic injection of LPS aggravated ROT neurotoxicity, as evidenced by a decreased moto-activity in the Rotarod test, pathology lesions in the substantia nigra, reduced brain dopamine and dopamine metabolites, increased inflammatory mediators such as TNF α , IL-1 β , iNOS, and COX2, as well as the activation of p-ERK1/2, p-38, and p-JNK signaling pathways (Huang et al. 2017). The current study further demonstrated that LPS plus ROT decreased non-motor activity in Elevated Plus Maze and Open Field tests, decreased mitochondrial complex 1 activity, and importantly, disrupted the circadian clock gene expression, as evidenced by the decreased expression of circadian clock core genes (Bmal1, Clock, Npas2), feedback control genes (Per1, Per2, Cry1, Cry2), and clock target genes (Dbp, Nr1d1) were decreased at the transcriptional and/or translational levels. These novel findings highlighted the importance of circadian rhythm disruption in the pathogenesis of PD.

It is well known that PD is a chronic and progressive neurodegenerative disease exacerbating with time (Braak et al. 2004). It is becoming increasingly evident that chronic neuroinflammation is a mechanism responsible for PD (Block et al. 2007). Peripheral/systemic inflammatory stress can also manifest in dopaminergic neuron loss likely through infiltration of peripheral leukocytes (Stojkowska et al. 2015; Gao et al. 2008). It has been reported that a single intraperitoneal LPS administration resulted in microglial activation and caused substantia nigra dopaminergic neuron loss 7 months post-injection in mice (Qin et al. 2007, 2013). This scenario in mice was duplicated in rats in the present study, another commonly used animal species in PD research, in the present study. LPS alone produced chronic neuroinflammation in SD rats in a dose-dependent manner, as evidenced by microglia activation (Iba1 staining) and dopaminergic neuron loss (TH staining) in a confocal image analysis (Figure 1). Pre-existed chronic neuroinflammation produced by a single injection of LPS in mice could aggravate other toxic effects of neurotoxicants, such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Byler et al. 2009), α -synuclein (Zhang et al. 2018), and DSP-4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine), a selective noradrenergic neurotoxin (Jiang et al. 2015). The same scenario was also applied in rats, as low-grade inflammation elicited by LPS potentiated low doses of ROT in producing neurotoxicity (Huang et al. 2017).

ROT is a pesticide in the environment, and its contamination is a risk factor of PD (Brown et al. 2006; Wang et al. 2011). ROT is used as a tool to produce PD animal models (Cannon et al. 2009; Sharma et al., 2013; Johnson and Bobrovskaya 2015). The present study demonstrated that a low dose of ROT (0.5 mg/kg) decreased mitochondrial complex 1 activity in the brain by 29% but in combination of LPS by 46%, fortifying that low-grade neuroinflammation from a single injection of LPS could potentiate the neurotoxic effects of ROT (Huang et al. 2017), and mitochondrial dysfunction is the major mechanism of ROT neurotoxicity.

Non-motor symptoms such as depression, constipation, sleep disorders and circadian rhythm dysfunction are frequently seen in PD patients (Tadaiesky et al. 2008; Willison et al. 2013; Schapira et al. 2017). In the present study, PD rats displayed the reduced exploratory activity and preferred to stay in local area in the open field test. In the elevated combined with maze test, PD rats preferred to stay in closed arms suggesting anxiety-like behaviors. In the Y

maze test, the successful spontaneous alternations were significantly decreased, indicating the impaired ability of learning and memory. Thus, non-motor syndromes are important indicators for ascending nature of PD progression (Braak et al. 2004).

Circadian system has recently been proposed a novel diagnostic and therapeutic target in PD and neurodegenerative disorders (Videnovic and Willis 2016), and we have shown that repeated manganese (Mn) administration in rats produced neurodegeneration and disruption of circadian of circadian clock genes in the brain and liver (Li et al. 2017b). Circadian rhythm disruption is a major non-motor symptom in PD patients (Schapira et al. 2017; Willison et al. 2013). In ROT-induced PD rat model, circadian rhythms were disrupted (Lax et al. 2012). Circadian rhythms are controlled and maintained by a series of clock genes (Mohawk et al. 2012; Buhr and Takahashi 2013; Partch et al. 2014). Clock, Npas2 and Bmal1 are the transcriptional activators of circadian clock system, which form a heterodimer to activate the feedback control genes Per and Cry (Buhr and Takahashi 2013; Partch et al. 2014). In PD patients, sleep dysfunction seen in early Parkinson's disease may reflect a more fundamental pathology in the molecular clock underlying circadian rhythms (Breen et al. 2014; Verwey et al. 2016), and decreased expression of Bmal1 (Cai et al. 2010) and Bmal2 (Ding et al. 2011) in leukocytes of PD patients was reported. Consistent with these observations, the expression of core clock genes Bmal1 and clock was decreased in a MPTP mouse model of PD (Hayashi et al. 2013). Similarly, levels of Bmal1 were also decreased in rotenone model of PD (Mattam and Jagota 2015). In addition, genetic polymorphisms in Arntl (Bmal1) as well as the promoter methylation of Npas2 gene (Lin et al. 2012) were found in PD patients, implying the importance of circadian clock in PD. The present study found that expressions of mRNA and protein of Bmal1, Clock and Npas2 all were decreased in PD rat model.

The activated feedback control genes Per and Cry products accumulate, dimerize, and form a complex to interact with BMAL1-CLOCK, repressing their own transcription (Buhr and Takahashi 2013; Partch et al. 2014). In animal PD models, unilateral depletion of dopamine by 6-OHDA injection into the medial forebrain results in blunting of daily expression of the clock protein PER2 in the dorsal striatum of rats (Hood et al. 2010). In ROT-induced PD rat model, levels of Per1 and Cry1 were decreased (Mattam and Jagota 2015). In a MPTP-treated mouse model of PD, expressions of Per1, Per2, Cry1 showed a decreased amplitude (Hayashi et al. 2013). Circadian rhythms of rest/activity of MitoPark mice are severely disturbed, which may be related with the loss of the midbrain dopaminergic neurons (Fifel and Cooper 2014). Importantly, rhythms in clock gene expression in the dorsal striatum are sensitive to changes in dopamine release (Verwey et al. 2016). Circadian disruption by triggering a robust neuroinflammatory reaction and degeneration of the nigral-dopaminergic neuronal system exacerbates process of PD (Lauretti et al. 2016). Consistent with PD patients and PD models, our presented study also found disrupted expressions of circadian clock feedback genes Per1 and Per2 in PD rat model, similar to our recent publication in Mn-induced PD-like neurodegeneration (Li et al. 2017b).

BMAL1-CLOCK targeted genes include Nr1d1, Dbp, and many others such as ROR, Nfil3, and Tef (Buhr and Takahashi 2013; Partch et al. 2014). In a MPTP-treated mouse model of PD, the expression of Rev-erba (Nr1d1) was significantly decreased which consistent with

our result. Dbp is a circadian PAR-domain basic leucine zipper-transcription factor, which is controlled directly by circadian clock core genes. In addition, our previous study found dysregulation of Dbp expression also occurred in human hepatocellular carcinoma (Li et al. 2017a) and in Manganism rats (Li et al. 2017b). For PD animal models, the presented study first reported the dysregulation of Dbp expression. The present study demonstrated circadian clock targeted gene Nr1d1 and Dbp expressions decreased both in mRNA and protein levels. However, in Manganism rats, the mRNA expression of Nr1d1 and Dbp was increased (Li et al. 2017b). This discrepancy could be due to different models (LPS plus ROT vs Mn), and whether the increased expression of clock target genes are unique to Mn intoxication warrants further investigation.

Circadian desynchronization influences normal patterns of day-night rhythms such as sleep and alertness cycles, rest, and activity cycles (Musiek and Holtzman 2016), as observed in PD (Chahine et al. 2016; Li S et al. 2017). Circadian disruption is a common, yet mostly unrecognized, pathologic mechanism of neurodegenerative disease (Smolensky et al. 2016), and may actually drive pathogenesis early in the course of neurodegenerative diseases (Musiek and Holtzman 2016; Willison et al. 2013). Thus, an understanding of circadian clock gene dysregulation in PD not only provides a biomarker of the disease, but also could provide a chrono-therapeutic intervention target (Dallman et al. 2014; Videnovic and Willis 2016).

In summary, this study demonstrated the expression of circadian clock genes and proteins was decreased in PD rat model induced by LPS combined with ROT, implying circadian disruption contributes to PD symptoms, and is an important non-motor manifestation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

LPS induced chronic neuroinflammation in rats. Adult male SD rats were given injections of saline, LPS 2.5, 5, and 7.5 mg/kg, ip, and 3 months later, the brains were collected for double stain for dopaminergic neurons (TH-stain, red) and microglia (Iba1-stain, green) (A), and their quantitative cell counts were expressed as % of Controls as described previously (Liu et al. 2008) (B). Values are the mean $\pm$ SEM (n = 3).

*Significantly different from the control group, $p < 0.05$.

Figure 2.

Effects of LPS combined with ROT on non-motor behavior. Rats were given LPS (5 mg/kg, i.p. x1), and 7 months later received 20 injections of rotenone (0.5 mg/kg, s.c., 5 time/week for 4 weeks), and the tests was performed 10 days after the last dose of rotenone. A, Open-field traveling distance; B, Open-field central/local activity ration; C. Open arm/closed arm in elevated combined with maze test; D. Correct alternative rate in the Y maze test. Values are the mean ± SEM (n = 6–9).

*Significantly different from the control group, $p < 0.05$.

Figure 3.

Effects of LPS and ROT on the activity of mitochondria complex 1 in cortex. Values are expressed as the percentage of controls, mean $\pm$ SEM (n = 6–9).

*Significantly different from the control group, $p < 0.05$.

Figure 4.

Effects of LPS and ROT on the expression of circadian clock core genes and proteins. Rats were given LPS (5 mg/kg, i.p. x1), and 7 months later 20 injections of rotenone (0.5 mg/kg, s.c., 5 time/week for 4 weeks). Two weeks after the last ROT treatment, rats were sacrificed and cortex was collected for analysis. A, mRNA expression of circadian clock core genes; B, The expression of BMAL1 protein; C, The expression of CLOCK protein. Values were mean $\pm$ SEM (n = 6–9).

*Significantly different from the control group,

#Significantly different from the LPS group, $p < 0.05$.

Figure 5.

Effects of LPS and ROT on the expressions of circadian clock feedback gene Per1, Per2, Cry1, and Cry2. Rats were given LPS (5 mg/kg, i.p. x1), and 7 months later 20 injections of rotenone (0.5 mg/kg, s.c., 5 time/week for 4 weeks). Two weeks after the last ROT treatment, rats were sacrificed and cortex was collected for analysis. Values are mean $\pm$ SEM (n = 6–9).

*Significantly different from the control group,

#Significantly different from the LPS group, $p < 0.05$.

Figure 6.

Effects of LPS and ROT on the expression of circadian clock target genes. Rats were given LPS (5 mg/kg, i.p. x1), and 7 months later 20 injections of rotenone (0.5 mg/kg, s.c., 5 time/week for 4 weeks). Two weeks after the last ROT treatment, rats were sacrificed and cortex was collected for analysis. A, mRNA expression of circadian clock target gene Nr1d1 and Dbp; B, the expression of NR1D1 protein; and C, the expression of DBP protein. Values are mean $\pm$ SEM (n = 6–9).

*Significantly different from the control group $p < 0.05$.

Table 1.

Primer sequences for rat circadian clock genes

Gene	Accession	Forward	Reverse
β -actin	NM-031144	AGCCATGTACGTAGCCATCC	ACCCTCATAGATGGGCACAG
Bmal1	NM_024362	AGCCAGTGAACGGGGAAATA	TATGCCAAAATAGCCGTCGC
Clock	NM_021856	CTGTTACATCAGCACGCCTC	TACTGCTTGGCTCTTCTGCT
Npas2	NM_001108214	CCTAGCCCCTCCTGTAATGG	AGGTTTCGTCAGCTACACACA
Cry1	NM_198750	ACGTGATAGGGAAGTGCACA	GTGCCTCAGTTTCTCCTCCT
Per1	NM_001034125	AAACAACAGCCACGGTTCTC	CACAAGCCGTTACATCGAGG
Per2	NM_031678	ACAGCTACACCACCCCTTAC	CAACACTGACACGGCAGAAA
Nr1d1	NM_001113422	AGCTGGTGAAGACATGACGA	TGCCATTGGAGCTGCTACTA
Dbp	NM_012543	GCGAGAAGTGCAAAATTGGC	CTATAGTCTGGGCGAGTGGG