REPORT

Acute Restraint Stress Augments 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine Neurotoxicity *via* Increased Toxin Uptake into the Brain in C57BL/6 Mice

Yasuhide Mitsumoto¹ · Atsushi Mori²

Received: 30 October 2017 / Accepted: 4 January 2018 / Published online: 9 July 2018
© Shanghai Institutes for Biological Sciences, CAS and Springer Nature Singapore Pte Ltd. 2018

Abstract As an environmental risk factor, psychological stress may trigger the onset or accelerate the progression of Parkinson's disease (PD). Here, we evaluated the effects of acute restraint stress on striatal dopaminergic terminals and the brain metabolism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which has been widely used for creating a mouse model of PD. Exposure to 2 h of restraint stress immediately after injection of a low dose of MPTP caused a severe loss of striatal dopaminergic terminals as indicated by decreases in the dopamine transporter protein and dopamine levels compared with MPTP administration alone. Both striatal 1-methyl-4-phenylpyridinium ion (MPP⁺) and MPTP concentrations were significantly increased by the application of restraint stress. Striatal monoamine oxidase-B, which catalyzes the oxidation of MPTP to MPP⁺, was not changed by the restraint stress. Our results indicate that the enhanced striatal dopaminergic terminal loss in the stressed mice is associated with an increase in the transport of neurotoxin into the brain.

Keywords MPTP · MPP⁺ · Mouse model · Restraint stress · Dopaminergic neuron · Degeneration · Dopamine transporter · Dopamine

Introduction

Parkinson's disease (PD) is a chronic and progressive neurodegenerative disorder characterized by the demise of dopaminergic neurons in the substantia nigra pars compacta and the concomitant reduction of dopamine (DA) in axon terminals in the striatum [1]. The pathogenesis of PD in most cases is unclear, but it is thought to result from the interaction between genetic and environmental causes [2, 3]. Physical and emotional traumas have been considered as factors in the etiology and pathophysiology of this disorder [4]. For example, when patients with PD are exposed to severe and long-term stresses, the symptoms are remarkably worsened [5, 6]. On the other hand, a strong positive association between depression caused by stress and the subsequent incidence of PD has been reported [7]. However, the bases of these stress-induced impairments in this disease have not been elucidated.

In the mouse model of PD created by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), several studies have indicated that emotional stress is involved in the vulnerability of striatal dopaminergic neurons to the toxin. For instance, augmentation of the toxin-induced neurotoxicity by chronic mild stress has been reported [8]. Chronic restraint stress also exacerbates the motor deficit and neuroinflammation in the MPTP mouse model [9]. Furthermore, chronic stress, as reflected by elevated glucocorticoid levels, promotes a pro-inflammatory state, activates microglia, and ultimately promotes the death of dopaminergic neurons in the substantia nigra [10].

However, the effects of acute stress on neurotoxicity and the brain metabolism of MPTP have not been elucidated. We therefore investigated the effects of an experimental stress on dopaminergic terminals and the brain metabolism

✉ Yasuhide Mitsumoto
y-mitsumoto@hokuriku-u.ac.jp

¹ Laboratory of Alternative Medicine and Experimental Therapeutics, Department of Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Hokuriku University, Ho-3 Kanagawa-machi, Kanazawa, Ishikawa 920-1181, Japan

² Department of Perioperative Management System, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan

of MPTP in C57BL/6 mice treated with a low dose of the neurotoxin.

Materials and Methods

Male C57BL/6 mice (8 weeks old, Charles River Japan, Atsugi, Japan) were housed at an ambient temperature of $23 \pm 2^\circ\text{C}$ under a 12 h light/12 h dark cycle (lights on at 07:00) with free access to food and water. All animal procedures were performed in accordance with the institutional guidelines and approved by the Animal Care Committee at Hokuriku University. Acute restraint stress was applied in ventilated cylinder tubes ($\phi 3 \times 12$ cm) that allowed little movement. After introducing a mouse into a tube, the tube was placed in vertical position with the mouse head upward, and left for 2 h. MPTP hydrochloride (Sigma–Aldrich, St Louis, MO) was dissolved in saline and injected intraperitoneally at a dose of 20 mg/kg free base. The dose used here was chosen because of the weak acute toxicity, according to previous reports [11].

Three days after MPTP administration, the striata of mice were collected. The left striatum was used for western blotting and the right striatum for high-performance liquid chromatography (HPLC). The left striatal tissues were homogenized and the protein concentration determined using a BCA Protein Assay Kit (Cat # 23227, Thermo Scientific, Rockford, IL). For western blotting, the homogenates were solubilized with Laemmli's sample buffer and subjected to 10% SDS polyacrylamide gel electrophoresis at 20 μg protein per lane. The gels were then transferred onto polyvinylidene difluoride membranes. Blots were incubated with a rat monoclonal antibody against the dopamine transporter (DAT) (MAB369, Millipore, Temecula, CA). The mouse monoclonal antibody against the actin (MAB1501, Millipore) was used as a loading control. The membranes were incubated with a secondary antibody, and processed for enhanced chemiluminescent autoradiography (Amersham ECL, GE Healthcare KK, Tokyo, Japan). Densitometric analysis was performed to quantify the relative protein level against loading control and expressed as a percentage of control. The DA content was measured using an HPLC–electrochemical detection system according to the previously-described method [11, 12]. The right striatal tissues were homogenized in 0.1 N perchloric acid containing isoproterenol as an internal standard. The homogenates were centrifuged at $20,000 \times g$ for 15 min at 4°C , and the DA content of the supernatant was measured. Striatal DA levels were calculated as ng/g tissue wet weight and expressed as a percentage of the control level. The measurements of striatal MPTP and 1-methyl-4-phenylpyridinium ion (MPP⁺) levels were performed as described by Desole

et al. [13] at several time points after MPTP injection. The striatal tissues were homogenized and centrifuged. MPP⁺ or MPTP content in the supernatant was measured using an HPLC system equipped with an UV detector, which was set to 295 nm for MPP⁺ and 245 nm for MPTP detection. Both striatal MPTP and MPP⁺ levels were expressed as ng/g tissue wet weight. Monoamine oxidase (MAO)-B activity in striatal tissue homogenates was assayed by a fluorometric method using benzylamine as the substrate [14] and expressed as a percentage of the control level.

The results are expressed as mean $\pm$ SEM. The statistical significance of differences was assessed by the two-tailed *t*-test or two-way analysis of variance (ANOVA) followed by Dunnett's test. The differences were considered significant at $P < 0.05$. All statistical analyses were performed using the Statistical Analysis System software (SAS Institute Japan, Ltd., Tokyo, Japan).

Results and Discussion

Exposure to 2 h of restraint stress immediately after MPTP injection caused a severe loss of striatal nerve terminals as indicated by lower DAT protein as well as DA levels than in mice given MPTP alone (Fig. 1A–C). The restraint stress alone did not affect the striatal DAT protein and DA levels. These results suggested that restraint stress augments the dopaminergic neurotoxicity of MPTP. Interestingly, among different starting times of the 2-h restraint stress (0, 2, 4, and 6 h after MPTP injection), MPTP toxicity was only enhanced in the 0–2 h range (data not shown). In this study, we chose a low dose of MPTP, because the influence of restraint stress might have been masked if severe MPTP toxicity was induced.

The neurotoxic effects of MPTP are thought to be mediated by its metabolite MPP⁺, which results from the oxidation of MPTP by MAO-B in glial cells. MPP⁺ has a high affinity for the DAT, and causes mitochondrial dysfunction and oxidative stress [15]. Therefore, it was important to investigate whether the restraint stress affects the striatal concentrations of MPP⁺ and MPTP. Both concentrations were significantly increased by the application of restraint stress (Fig. 2A, B). However, the activity of striatal MAO-B, which catalyzes the oxidation of MPTP to MPP⁺, was not changed by restraint stress (Table 1). These results indicated that the increase of MPP⁺ production in the striatum by restraint stress was not due to activation of a metabolic process of MPTP.

In the present study, exposure to 2 h of restraint stress immediately after MPTP injection caused severe striatal dopaminergic terminal loss compared with MPTP treatment alone. In addition, we found increases in both striatal MPP⁺ and MPTP concentrations, which were not due to

Fig. 1 Effects of acute restraint stress on striatal DAT protein and DA levels in MPTP-treated C57BL/6 mice. **A** Left striatal homogenates were prepared and DAT proteins were detected by Western blotting. Actin served as a loading control. $n = 8$ mice/group. **B** Densitometric analysis of the protein bands; DAT protein levels are expressed as percentage of control (mean $\pm$ SEM). **C** Right striatal DA measured by HPLC-ECD; data expressed as percentage of control (mean $\pm$ SEM). $**P < 0.01$ vs Control, $##P < 0.01$ vs MPTP (two-tailed t -test).

Fig. 2 Changes in striatal MPP⁺ (A) and MPTP (B) induced by acute restraint stress in MPTP-treated C57BL/6 mice. MPTP (20 mg/kg, i.p.) was injected and animals were sacrificed at various time points

with (solid line) or without (dashed line) restraint stress. Data are presented as mean $\pm$ SEM ($n = 6$). $\&\&P < 0.01$ vs Control (two-way ANOVA, Dunnett's test).

Table 1 Effects of acute restraint stress on striatal MAO-B activity in MPTP-treated mice.

	<i>N</i>	Striatal MAO-B activity (% of Control)	vs. Control
Control	5	100 $\pm$ 2.1	<i>N.S.</i>
MPTP	5	96 $\pm$ 1.4	<i>N.S.</i>
MPTP + Restraint stress	5	97 $\pm$ 2.1	<i>N.S.</i>
Restraint stress	5	98 $\pm$ 1.5	<i>N.S.</i>

Mice were given a single injection of MPTP (20 mg/kg, i.p.) and immediately exposed to restraint stress for 2 h. The measurements were made at 2 h after MPTP injection. Data are presented as mean $\pm$ SEM and expressed as percentage of control. *N.S.*, not statistically significant vs control.

MAO-B activation, in the stressed mice. The hypothalamic-pituitary-adrenal axis is critical for homeostasis in response to environmental stressors [16, 17]. This axis includes the hypothalamic paraventricular nucleus (secretes corticotrophin-releasing factor (CRF) and arginine vasopressin), the pituitary gland (releases corticotrophin triggered by CRF and arginine vasopressin), and the adrenal cortex (secretes glucocorticoids) [18]. Esposito *et al.* reported that CRF is associated with the regulation of blood-brain barrier (BBB) permeability induced by restraint stress [19]. Taking this into consideration, we propose that the enhanced MPTP neurotoxicity in stressed mice is associated with an increase in the neurotoxin transport *via* changes in CRF-induced BBB permeability. However, the influence of acute stress on BBB permeability is controversial. *In vivo* animal studies have reported that stress increases the BBB permeability to inorganic dyes like Evans Blue and sodium fluorescein [20, 21]. On the other hand, several other reports have not been able to detect effects of stress on BBB permeability [22, 23]. Therefore, further studies are needed to clarify the relationship between restraint stress and MPTP transport through the BBB and the blood-cerebrospinal fluid barrier.

The symptoms of patients with PD are often worsened by various stresses [24, 25]. We previously reported that in Mg^{2+} -deficient mice, emotional behaviors such as depression- and anxiety-related behaviors, appear to increase the susceptibility to MPTP [26]. Our data showed that the decrease of striatal DA level by a low dose of MPTP only occurs in Mg^{2+} -deficient mice that display depression- and anxiety-related behaviors, but not in control mice. Therefore, we concluded that the brain's responses to emotion-inducing situations influence susceptibility to the dopaminergic neurotoxin. Based on the findings obtained here, the uptake of MPTP into the brain may be altered in Mg^{2+} -deficient mice.

In conclusion, the results of the present study demonstrated that acute restraint stress augmented the neurotoxicity of a low dose of MPTP in C57BL/6 mice. Increased toxin uptake into the brain is thought to be part of the mechanism underlying the enhanced neurotoxicity. To our knowledge, this is the first evidence showing that acute restraint stress increases the transport of MPTP into the brain. Although the pathogenesis of PD in most cases is unclear, individuals exposed to environmental poisons that might be toxic to nigrostriatal dopaminergic neurons, may have to avoid excessive stress. Further studies are needed to test this hypothesis.

Acknowledgements This work was supported by the Specific Research Foundation of Hokuriku University (250100).

References

- Olanow CW, Tatton WG. Etiology and pathogenesis of Parkinson's disease. *Annu Rev Neurosci* 1999, 22: 123–144.
- Mizuno Y, Yoshino H, Ikebe S, Hattori N, Kobayashi T, Shimoda-Matsubayashi S, *et al.* Mitochondrial dysfunction in Parkinson's disease. *Ann Neurol* 1998, 44: S99–S109.
- Li S, Le W. Milestones of Parkinson's disease research: 200 years of history and beyond. *Neurosci Bull* 2017, 33: 598–602.
- Djamshidian A, Lees AJ. Can stress trigger Parkinson's disease? *J Neurol Neurosurg Psychiatry* 2014, 85: 878–881.
- Snyder AM, Stricker EM, Zigmond MJ. Stress-induced neurological impairments in an animal model of parkinsonism. *Ann Neurol* 1985, 18: 544–551.
- Smith AD, Castro SL, Zigmond MJ. Stress-induced Parkinson's disease: a working hypothesis. *Physiol Behav* 2002, 77: 527–531.
- Schuurman AG, van den Akker M, Ensink KT, Metsemakers JF, Knottnerus JA, Leentjens AF, *et al.* Increased risk of Parkinson's disease after depression: a retrospective cohort study. *Neurology* 2002, 58: 1501–1504.
- Janakiraman U, Manivasagam T, Justin Thenmozhi A, Dhanalakshmi C, Essa MM, Song BJ, *et al.* Chronic mild stress augments MPTP induced neurotoxicity in a murine model of Parkinson's disease. *Physiol Behav* 2017, 173:132–143.
- Lauretti E, Di Meco A, Merali S, Praticò D. Chronic behavioral stress exaggerates motor deficit and neuroinflammation in the MPTP mouse model of Parkinson's disease. *Transl Psychiatry* 2016, 6: e733.
- de Pablos RM, Herrera AJ, Espinosa-Oliva AM, Sarmiento M, Muñoz MF, Machado A, *et al.* Chronic stress enhances microglia activation and exacerbates death of nigral dopaminergic neurons under conditions of inflammation. *J Neuroinflammation* 2014, 11: 34.
- Mori A, Ohashi S, Nakai M, Moriizumi T, Mitsumoto Y. Neural mechanisms underlying motor dysfunction as detected by the tail suspension test in MPTP-treated C57BL/6 mice. *Neurosci Res* 2005, 51: 265–274.
- Mitsumoto Y, Mori A, Ohashi S, Nakai M, Moriizumi T. Differential effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in the olfactory bulb and the striatum in mice. *Neurosci Res* 2005, 51: 111–115.
- Desole MS, Esposito G, Fresu L, Migheli R, Enrico P, Miele M *et al.* Correlation between 1-methyl-4-phenylpyridinium ion (MPP^{+}) levels, ascorbic acid oxidation and glutathione levels in the striatal synaptosomes of the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated rat. *Neurosci Lett* 1993, 161: 121–123.
- Suzuki O, Noguchi E, Yagi K. A simple fluorometric assay for type B monoamine oxidase activity in rat tissues. *J Biochem* 1976, 79: 1297–1299.
- Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. *Neuron* 2003, 39: 889–909.
- McEwen BS. Physiology and neurobiology of stress and adaptation: central role of the brain. *Physiol Rev* 2007, 87: 873–904.
- Lupien SJ, McEwen BS, Gunnar MR, Heim C. Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nat Rev Neurosci* 2009, 10: 434–445.
- Goncharova ND. Stress responsiveness of the hypothalamic-pituitary-adrenal axis: age-related features of the vasopressinergic regulation. *Front Endocrinol* 2013, 4: 26.
- Esposito P, Chandler N, Kandere K, Basu S, Jacobson S, Connolly R, *et al.* Corticotropin-releasing hormone and brain mast cells regulate blood-brain-barrier permeability induced by acute stress. *J Pharmacol Exp Ther* 2002, 303: 1061–1066.

20. Esposito P, Gheorghe D, Kandere K, Pang X, Connolly R, Jacobson S, *et al.* Acute stress increases permeability of the blood-brain-barrier through activation of brain mast cells. *Brain Res* 2001, 888: 117–127.
21. Sarmiento A, Borges N, Lima D. Influence of electrical stimulation of locus coeruleus on the rat blood-brain barrier permeability to sodium fluorescein. *Acta Neurochir (Wien)* 1994, 127: 215–219.
22. Ohata M, Fredericks WR, Sundaram U, Rapoport SI. Effects of immobilization stress on regional cerebral blood flow in the conscious rat. *J Cereb Blood Flow Metab* 1981, 1: 187–194.
23. Roszkowski M, Bohacek J. Stress does not increase blood-brain barrier permeability in mice. *J Cereb Blood Flow Metab* 2016, 36: 1304–1315.
24. Urakami K, Masaki N, Shimoda K, Nishikawa S, Takahashi K. Increase of striatal dopamine turnover by stress in MPTP-treated mice. *Clin Neuropharmacol* 1988, 11: 360–368.
25. Li S, Wang Y, Wang F, Hu LF, Liu CF. A new perspective for Parkinson's disease: circadian rhythm. *Neurosci Bull* 2017, 33: 62–72.
26. Muroyama A, Inaka M, Matsushima H, Sugino H, Marunaka Y, Mitsumoto Y. Enhanced susceptibility to MPTP neurotoxicity in magnesium-deficient C57BL/6 N mice. *Neurosci Res* 2009, 63: 72–75.