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Single low doses of MPTP decrease tyrosine hydroxylase expression in the absence of overt neuron loss

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ABSTRACT

Parkinson's disease (PD) is the second most common age-related neurodegenerative disease. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a prototypical neurotoxicant used in mice to mimic primary features of PD pathology including striatal dopamine depletion and dopamine neuron loss in the substantia nigra pars compacta (SNc). In the literature, there are several experimental paradigms involving multiple doses of MPTP that are used to elicit dopamine neuron loss. However, a recent study reported that a single low dose caused significant loss of dopamine neurons. Here, we determined the effect of a single intraperitoneal injection of one of three doses of MPTP (0.1, 2 and 20 mg/kg) on dopamine neurons, labeled by tyrosine hydroxylase (TH^+), and total neuron number ($Nissl^+$) in the SNc using unbiased stereological counting. Data reveal a significant loss of neurons in the SNc (TH^+ and $Nissl^+$) only in the group treated with 20 mg/kg MPTP. Groups treated with lower dose of MPTP (0.1 and 2 mg/kg) only showed significant loss of TH^+ neurons rather than TH^+ and $Nissl^+$ neurons. Striatal dopamine levels were decreased in the groups treated with 2 and 20 mg/kg MPTP and striatal terminal markers including, TH and the dopamine transporter (DAT), were only decreased in the groups treated with 20 mg/kg MPTP. These data demonstrate that lower doses of MPTP likely result in loss of TH expression rather than actual dopamine neuron loss in the SN. This finding reinforces the need to measure both total neuron number along with TH^+ cells in determining dopamine neuron loss.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease among the elderly in which the individual suffers from motor impairments. Nearly 1% of the world population suffers from PD, clinically diagnosed with rigidity, resting tremor, and bradykinesia (Cannon and Greenamyre, 2013; Klingelhoefer and Reichmann, 2015). Pathophysiological features of PD consist of dopaminergic cell degeneration in the substantia nigra pars compacta (SNc), striatal dopamine (DA) depletion and protein aggregates compromised of alpha-synuclein and ubiquitin (Klingelhoefer and Reichmann, 2015; Gibb and Lees, 1991; Rodriguez-Oroz et al., 2009).

Multiple genetic and toxin animal models of PD have been developed to study the underlying mechanisms of this complex neurodegenerative disease. Transgenic mouse models of multiple genes involved in the development of PD such as alpha synuclein, Parkin, PINK, LRRK2, DJ-1 have been proven to be beneficial in the study of PD, but typically do not reproduce the full manifestation of PD phenotypes (Masliah et al., 2000; Yacoubian et al., 2008; Sommer et al., 2000; Goldberg et al., 2003; Gispert et al., 2009; Tong et al., 2009; Andres-Mateos et al., 2007). The case of frozen addicts and the discovery of the involvement of MPTP in the development of Parkinson like symptoms in these individuals triggered the research using MPTP as a neurotoxicant to mimic PD in animal models such as nonhuman primates and mice (Langston et al., 1984). MPTP is the most widely used prototypical neurotoxicant to study the underlying mechanisms of PD (Jackson-Lewis et al., 1995; Bezard et al., 1997; Petroske et al., 2001). High doses of MPTP result in DA neuron loss in the SNc and striatal DA depletion. Thus, MPTP animal models are valuable tools to study PD, as they mimic many aspects of the pathophysiology observed in PD.

Numerous models incorporating cumulative MPTP doses ranging between 20–250 mg/kg, either as several doses in one day or over the course of several days to a week, have been developed in order to study the neurodegeneration of the nigrostriatal pathway (Jackson-Lewis et al., 1995; Bezard et al., 1997; Dovero et al., 2016). Although multiple high doses (10–

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30 mg/kg) of MPTP are generally required to cause DA neuron loss, a recent study reported that single low doses of MPTP (0.1–2 mg/kg) caused a loss of tyrosine hydroxylase positive (TH^+) cells in young OF1 mice (Dovero et al., 2016). The purpose of this study was to determine the effects of lower single MPTP doses on dopamine neuron loss and dopaminergic neurochemistry and assess whether similar results are obtained in C57BL/6J mice, the best-characterized and most common strain of mice used for MPTP studies.

2. Materials and methods

2.1. Chemicals

MPTP-HCl was purchased from Sigma–Aldrich (Milwaukee, WI, USA). Polyclonal rabbit anti-TH (cat#AB152) was purchased from EMD Millipore (Billerica, MA, USA). Monoclonal rat anti-DAT (cat# AB5990) and α-tubulin antibody (cat#AB80799) were purchased from Abcam (Cambridge, UK) and polyclonal rabbit anti-VMAT2 was a gift from Dr. Gary Miller at Emory University. Alexafluor secondary antibodies (anti-rabbit and anti-mouse) were purchased from Thermo-Fisher Scientific (Waltham, MA). Horseradish peroxidase conjugated secondary antibodies were purchased from Bio-Rad (Hercules CA). Biotinylated secondary antibody and 3,3-diaminobenzidine–peroxidase (DAB) including nickel enhancer for immunohistochemistry were purchased from Vector laboratories (PK-6100, SK-4100, Burlingame, CA). Monoamine standards for high performance liquid chromatography-electrochemical detection (HPLC-ECD) analysis for dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were purchased from Sigma–Aldrich (Milwaukee, WI, USA). All other chemicals were purchased from Sigma–Aldrich or Thermo-Fisher unless specifically noted.

2.2. Animals

C57BL/6J male mice (3–4 months old) were obtained from Jackson laboratories (Bar Harbor, ME). Animals were group housed with a maximum of three animals per cage and acclimated to the vivarium for a week prior to initiation of the study. Animals were kept in a normal 12:12 h light/dark cycle with free access to food and water. All procedures were conducted in accordance with the National Institutes of Health's Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the Northeast Ohio Medical University.

2.3. Experimental design and treatment

Mice ($n = 5$–6 per group) received a single intraperitoneal (i.p.) injection of either saline or MPTP in a total volume of 100 μl. The three MPTP doses of 0.1, 2 and 20 mg/kg were selected based on the study conducted by Dovero and colleagues (Dovero et al., 2016). Seven days after dosing, mice were euthanized. The whole midbrain and right hemisphere of the striatum were harvested and immersion fixed in 4% paraformaldehyde (PFA) for 72 h. After fixation, the brain samples were placed in 30% sucrose and stored at 4 °C until immunohistochemical processing. Striatum from the left hemisphere was dissected out from the forebrain, frozen in liquid nitrogen and stored at −80 °C until HPLC and western blotting was performed. During the course of study, all animals were weighed twice, once before dosing and a week after dosing (before they were euthanized).

2.4. Sample preparation

The dissected left striatum of each animal was sonicated in 500 μl of homogenizing buffer (320 mM sucrose, 5 mM HEPES and 1 μl/ml protease inhibitor cocktail; pH 7.4). Each sample was then aliquoted into two different tubes of 200 and 300 μl to be used in HPLC and western blot analysis, respectively.

2.5. HPLC with electrochemical detection of striatal neurochemistry

The aliquoted homogenates for HPLC were further diluted with 0.2 N perchloric acid at a 1:1 ratio and centrifuged at 14,000 × rpm for 20 min at 4 °C; supernatants were collected and filtered using a 0.45 micron filtered tube. The pellets were dried and kept for protein assay. HPLC analysis for catecholamines was performed essentially as described by our previous work (Yochum et al., 2014). Briefly, an aliquot of 10 μl of the supernatant was injected into the HPLC (Waters, Milford, MA, USA) for neurochemical analysis of DA and its metabolites, DOPAC and HVA. A cation exchange reverse column (MD-150 × 3.2 column, ESA Biosciences Inc.) was used to separate the components. Isocratic mobile phase (MD-TM mobile phase, Thermo-Fisher Scientific, Waltham, MA) containing 2.2 mM filtered NaCl adjusted to pH 3.3 pumped at a constant flow rate of 0.4 ml/min was used. The compounds were quantified by electrochemical detection using glossy carbon working electrode, 2.0 mm diameter in situ silver reference electrode (Flow cell, 2 mm GC, ISAC, waters, Milford, MA, USA). Quantification of DA and metabolites was made with reference to the standard curves obtained from individual standards (DA, DOPAC, and HVA) and were normalized to protein concentration measured by BCA kit (Thermo-Fisher Scientific, Waltham, MA).

2.6. Western immunoblots

Western blots were performed as described previously (Hossain and Richardson, 2011; Hossain et al., 2015). Briefly, the homogenates were centrifuged at 3500 × rpm for 5 min at 4 °C. The supernatant was collected and centrifuged at 14,000 × rpm for 45 min at 4 °C. The pellets were re-suspended in 100 μl of homogenization buffer supplemented with 0.1% protease inhibitor. Following protein quantification, 20 μg of protein sample was loaded per lane on 4–12% Tris-Bis NuPage gels (Invitrogen, Carlsbad, CA). After electrophoresis, proteins from the gels were transferred to a polyvinylidene difluoride membrane, using the iBlot transfer machine. The nonspecific protein-binding sites on the membrane were blocked with 7.5% nonfat dry milk in Tris-buffered saline (135 mM NaCl, 2.5 mM KCl, 50 mM Tris, and 0.1% Tween 20, pH 7.4). After blocking, the membrane was incubated in a rabbit polyclonal TH antibody (1:2000) in Tris-buffered saline with 2% nonfat dry milk overnight at 4 °C. The membrane was washed three times and incubated in a goat anti-rabbit horseradish peroxidase secondary antibody (1:20,000) for an hour at room temp. The specific antibody-bound protein was detected by chemiluminescence using an Alpha Innotech Fluorochem (San Leandro, CA) imaging and stored as a digital image. The membrane was then stripped for 15 min at 25 °C in Stripping Buffer (Thermo fisher Scientific, cat# P146430) and re-probed sequentially with a monoclonal antibody to the N-terminus of DAT (1:1000) and a polyclonal VMAT2 (1:1000) antibody. A monoclonal α-tubulin antibody (1:1000) was used to confirm equal loading of samples and the densitometric value for each sample was normalized to its associated α-tubulin value for statistical analysis.

2.7. Immunohistochemistry and unbiased stereology

Midbrains were coronally sectioned (40 μM) on a freezing, sliding microtome (HM 450 microtome, Thermo Fisher Scientific, Waltham, MA). The sections containing the substantia nigra were collected and kept in a cryoprotective solution (25% sucrose and