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Direct intranigral injection of dopaminochrome causes degeneration of dopamine neurons

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Abstract

Parkinson's disease (PD) is characterized by progressive neurodegeneration of nigrostriatal dopaminergic neurons leading to clinical motor dysfunctions. Many animal models of PD have been developed using exogenous neurotoxins and pesticides. Evidence strongly indicates that the dopaminergic neurons of the substantia nigra pars compacta (SNpc) are highly susceptible to neurodegeneration due to a number of factors including oxidative stress and mitochondrial dysfunction. Oxidation of DA to a potential endogenous neurotoxin, dopaminochrome (DAC), may be a potential contributor to the vulnerability of the nigrostriatal tract to oxidative insult. In this study, we show that DAC causes slow and progressive degeneration of dopaminergic neurons in contrast to 1-methyl-4-phenylpyridinium (MPP⁺), which induces rapid lesions of the region. The DAC model may be more reflective of early stresses that initiate the progressive neurodegenerative process of PD, and may prove a useful model for future neurodegenerative studies.

Keywords

Parkinson's disease; neurodegeneration; substantia nigra pars compacta; dopamine; oxidation

1. Introduction

Parkinson's disease is characterized by the degeneration of the dopaminergic neurons of the substantia nigra pars compacta (SNpc) that project to the striatum to facilitate coordinated and controlled movements [3, 15]. Motor symptoms of PD are attributed to a loss of dopamine (DA) signaling within the dorsal striatum [30, 32]. These symptoms do not appear until over 80% of nigral neurons are lost, making it very difficult to study the progression of the disease.

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Conflict of interest

The authors declare no conflicts of interest

Dopaminergic neurons of the SNpc are highly susceptible to oxidative stress and mitochondrial dysfunction [16, 27, 34]. The neurotransmitter DA may be an endogenous contributor to the vulnerability of nigrostriatal neurons to oxidative insult. DA oxidizes easily at physiological pH, either enzymatically or by autooxidation [18, 22, 50] and may be an endogenous contributor to the neurodegeneration of nigrostriatal neurons. DA oxidation produces dopaminochrome (DAC) [10, 21, 42, 50, 56], a component of neuromelanin, the substance that pigments SNpc neurons [17, 60]. We have previously reported that DAC is cytotoxic to mesencephalic cells in an oxidative stress-dependent manner [35, 36].

Several animal models of PD exist that are induced by systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, or paraquat, which cause degeneration of the nigrostriatal neurons through mitochondrial inhibition [5, 20, 26, 54]. Direct injection models alleviate the high mortality and variability of systemic models. Acute injections of 6-OHDA [9, 28] and 1-methyl-4-phenylpyridinium (MPP⁺; the reactive metabolite of MPTP) have long been used to specifically target the nigrostriatal dopaminergic pathway. However, the rapid lesions induced by these toxins differ from the progressive degeneration of the nigrostriatal neurons in PD.

Recent research has focused on animal models where damage occurs progressively to model earlier stages of the disease [6, 24, 45]. In line with those efforts, we propose that direct intranigral administration of DAC may better recapitulate the more progressive nature of PD. Paris et al. [43] have similarly proposed the use of aminochromes for research of early PD. In this study, we show that unilateral administration of DAC into the SNpc causes slow and progressive degeneration of dopaminergic neurons, in contrast to MPP⁺ that causes acute lesioning. The DAC model may be more reflective of early stresses that initiate the progressive neurodegenerative process of PD, and may prove a useful model for future therapeutic studies.

2. Materials and Methods

2.1. Experimental Animals

All animal experiments were conducted in accordance with the guidelines mandated in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of Saint Louis University Health Sciences Center. Adult male Sprague Dawley rats (Charles River Laboratories, MA, USA) weighing 250 – 300 g were used for all experiments. The rats were housed on a 12-h light–dark cycle. Access to food and water was provided *ad libitum*.

2.2. Dopaminochrome Preparation and Purification

DAC was freshly prepared for each experiment by oxidizing 40 mM DA in 0.9% sterile saline with equal volume of mushroom tyrosinase in 0.9% sterile saline at a concentration of 100 units activity/μL at 37°C for 1 min [21, 58]. This reaction yields a final concentration of 20 mM DAC. The DAC was separated from the tyrosinase by filtration through a 10,000 NMWL filter unit and was kept on ice until use. Conversion from DA to DAC was confirmed by wavelength scan from 200–600 nm and compared to the scan for DA. The

final concentration and complete conversion was further confirmed by HPLC-ED as previously described [41]. All compounds were obtained from Sigma-Aldrich (St. Louis, MO).

2.3. MPP⁺ preparation

MPP⁺ was freshly prepared for each experiment. All compounds were obtained from Sigma-Aldrich (St. Louis, MO). MPP⁺ was weighed and diluted in sterile 0.9% saline. All solutions for stereotaxic injections were spin-filtered through a centrifuge tube containing a 10,000 NMWL filter at 14,000xg for 15min.

2.4. Stereotaxic Intranigral Injection

Rats were anesthetized with intraperitoneal (i.p.) ketamine/xylazine (87:13 mg/Kg) and immobilized in a stereotaxic frame to conform to Paxinos and Watson's brain atlas. Borosilicate glass capillaries pulled to a diameter of 25 – 40 µm were used for pressure injection of the sterile solutions into the brain. Animals received a single injection of DAC (10 nmoles in 500 nL) into the left SN (–5.4 from bregma, +2.3 from midline, and –7.2 from dura) at a rate of 250 nL/min. An internal control of sterile 0.9% saline was injected into the right SNpc at equivalent volume. To minimize reflux up the injection tract, the needle remained in position for 8-10 minutes post-injection.

2.5. Immunohistochemistry

At the experimental end-point, rats were anesthetized with ketamine/xylazine and perfused intracardially with 4% paraformaldehyde. The brains were removed, post-fixed overnight, cryoprotected in 30% sucrose at 4°C, and cut via freezing microtome into 6 series of 40 µm thickness. Series were stored in a cryoprotectant solution consisting of 30% ethylene glycol and 30% sucrose. Antigen retrieval was performed using 1% sodium borohydride. Sections were incubated with the following primary antibodies: mouse anti-tyrosine hydroxylase (TH, 1:4000, BD Biosciences, overnight), mouse anti-NeuN (1:1000, Millipore, overnight). Secondary antibodies: biotinylated goat anti-mouse Ig (1:200, BD Pharmingen, 2hr). ABC reagent was then used (1:200, Vector Laboratories, 2hr), then the series were reacted for 1-5 minutes in Nickel-DAB solution containing 0.015% DAB, 0.4% nickel ammonium sulfate, and 0.003% hydrogen peroxide. The sections were visualized with an Olympus BX51 microscope and photographed using a DVC 2000C-oo-GE-MBF digital camera.

2.6. Cell Counts and Statistical Analysis

Sections selected for counting represented corresponding rostrocaudal levels and contained the same sets of structures in both experimental groups. The TH⁺ neurons in the SNpc were marked via NeuroLucida software (MBF Bioscience) and total counts were determined by NeuroLucida NeuroExplorer software (MBF Bioscience). TH⁺ immunoreactivity was quantified in the left (DAC) and right (saline) SNpc. A colleague, blinded to the treatment groups, verified the TH⁺ neuron counts. Counts for the DAC injected left SNpc were then compared to the saline injected control right SNpc side of the same rat and the results were expressed as percentage of the control side.

3. Results

3.1 MPP⁺ injection induces acute lesioning of the SNpc

Rats received injections of MPP⁺ (1-10µg, left side) and 0.9% saline (internal control, right side) stereotactically into the SNpc. Five days later, rats were intracardially perfused and brains were harvested and processed via immunohistochemistry (IHC) for TH. MPP⁺ fully lesioned TH⁺ neurons in the injected side (shown as black DAB staining) at all amounts administered when compared to the saline-injected internal control, with the exception of the lowest amount (1µg), which did not significantly reduce TH staining from control values (Figure 1).

3.2 DAC injection into the SNpc induces a progressive degeneration of the SNpc

Rats received injections of DAC (5 or 10 nmoles; left side) and saline (control; right side) stereotactically into the SNpc. Two, five, or ten days later the rats were intracardially perfused and brains were harvested and processed via IHC for TH to evaluate the neurodegenerative effects of DAC. Because DAC is brown in color, we used a nickel-DAB solution to generate black color at the site of immunoreactivity for TH. Two (not shown) or five days post SNpc injection of 10 nmoles of DAC, there was no reduction in TH immunoreactivity in the DAC injected side. Similarly, injection of 5 nmoles DAC did not cause a decrease in TH immunoreactivity after 10 days (Figure 2). However, 10 days post injection of 10 nmoles DAC, immunoreactivity for TH was significantly reduced in the DAC injected side and there was pronounced morphological damage to SNpc dendritic segments (Figure 3). No changes were observed in the striatum at any of these time-points (not shown).

4. Discussion

We demonstrate that administration of 10 nmoles DAC into the SNpc *in vivo* causes delayed and progressive degeneration of TH⁺ neurons 10 days after injection. In contrast, direct injection of MPP⁺ into the same coordinates induces acute lesioning of TH⁺ neurons, where higher amounts fully lesion the region and lower amounts cause little to no loss of TH immunostaining. We propose that direct injection of DAC into the SNpc may be an appropriate model for evaluating the early pathological changes of PD.

DAC is cytotoxic to mesencephalic cells in an oxidative stress-dependent manner [35, 36], inhibits mitochondrial respiration, and inactivates intracellular proteins [2, 13, 31, 57, 58, 62]. DAC is produced when DA is oxidized to quinone species that cyclize [21]. DAC is rapidly polymerized to form neuromelanin that pigments the SNpc [17, 60]. Neuromelanin may be neuroprotective by sequestering oxidized DA, proteins, and lipids from the cytosol to prevent cellular toxicity, however as neurons containing neuromelanin degenerate, neuromelanin is released into the extracellular space, inducing microglial activation and an enhanced inflammatory and oxidative state [59, 61]. This change in the effect of neuromelanin from beneficial to pathological may play a pivotal role in the enhanced susceptibility of nigrostriatal neurons to degenerate in PD. Therefore, DAC-induced neurodegeneration may be more relevant to understanding the pathology of idiopathic PD.

In fact it is interesting to note the fact that after 10 days there is a brown mark around the DAC injection site possibly indicative of neuromelanin formation.

MPTP produces rapid and irreversible parkinsonism in humans and non-human primates [19]. MPP⁺, the active metabolite of MPTP, is often directly injected into the SNpc of animals to specifically target the region and reduce mortality [8, 23, 39]. MPP⁺ is transported into dopaminergic neurons via the DA reuptake transporter [7, 25], accumulates in mitochondria interfering with oxidative phosphorylation [40, 49], leading to oxidative stress and apoptosis [46, 47]. MPP⁺ also induces dopaminergic neurotoxicity due to oxidation of intracellular DA [1, 37]. MPP⁺ is accumulated into synaptic vesicles by vesicular monoamine transporter 2 (VMAT2) [38, 52], which displaces stored DA to the cytosol. DA then undergoes robust efflux from neurons into the extracellular space mediated by DA uptake transporters (DAT) [33, 37, 51]. Choi et al. [12] reported abnormalities in the storage, DAT-mediated transport, and catabolic breakdown of DA in dopaminergic neurons treated with MPP⁺ and suggested that accumulation of cytosolic DA accounts for 30% of MPP⁺-mediated toxicity. These findings support a role for DA in the vulnerability of dopaminergic neurons to MPP⁺ and other oxidative insults as well.

Another common neurotoxin used in models of PD is 6-hydroxydopamine (6-OHDA), which is structurally similar to DA and has a high affinity for the DA uptake transporters [9, 11]. When injected directly into the SNpc, most concentrations destroy dopaminergic neurons within a few hours [28], although damage can continue to progress for several days [4, 29, 44, 63]. Some studies have attempted to induce partial lesions by reducing the amount of 6-OHDA injected into the SNpc, but this often leads to high variability and is unreliable [14, 55].

Direct injection of MPP⁺ in this study and others results in rapid and robust lesioning of TH-containing SNpc neurons, more closely resembling end-stage PD [48, 53]. In contrast, direct injection of DAC into the SNpc results in a delayed but significant loss of TH-containing neurons in the SNpc. DAC is a compound that is endogenously produced in the brain and is known to result in oxidative stress-induced neuronal damage. We propose that a DAC animal model provides a way to study the progression of pathophysiological changes associated with the development of neurodegeneration.

Acknowledgements

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Abbreviations

DAC	Dopaminochrome
MPP⁺	1-methyl-4-phenylpyridinium
SNpc	substantia nigra pars compacta

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Highlights

- Dopaminochrome was injected directly into the substantia nigra pars compacta
- Neurodegeneration of nigrostriatal neurons was compared between MPP⁺ and dopaminochrome-injected animals
- Dopaminochrome induced a slow and progressive degeneration of nigrostriatal neurons in the substantia nigra pars compacta

Figure 1. Direct injection of MPP⁺ into the SNpc induces severe lesioning

Immunostaining in the rat SNpc 5 days after MPP⁺ injection. Rats received intranigral injections of 2µL of MPP⁺ (left) or 0.9% sterile saline. Representative TH immunostaining of the SNpc injected with A) 10µg, B) 5µg, C) 3µg, D) 2µg, or E) 1µg MPP⁺, and saline shown.

Figure 2. Injection of 5 nmoles DAC does not result in loss of TH-containing neurons at day 10, neither does 10 nmoles DAC at day 5
Rats received intranigral injections of DAC (left) and 0.9% sterile saline. Representative TH immunostaining of the SNpc for DAC injected at 5 nmoles for 10 days (A, C) or 10 nmoles for 5 days (E, G). The images in C, D, G, and H indicate higher magnifications of the images in A, B, E, and F, respectively.

Figure 3. Injection of 10 nmoles DAC induces neurodegeneration of the SNpc by day 10
 TH immunostaining of the SNpc 10 days after DAC injection. A) Representative image at 4X magnification from a rat that received intranigral injections of DAC (10 nmoles, left) and 0.9% sterile saline. B) 10X magnifications of the DAC- and saline- injected SNpc. C) 20X magnifications of the DAC- and saline-injected SNpc. D) Total counts of TH+ neurons in DAC vs saline-injected SNpc shown.