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Research Report

Intranasally-administered deferoxamine mitigates toxicity of 6-OHDA in a rat model of Parkinson's disease

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ABSTRACT

Deferoxamine (DFO) has shown therapeutic promise for the treatment of Parkinson's disease (PD) as it has reduced both behavioral and biochemical deficits when injected into the brain of rodent models of PD. Intranasally administered DFO targets the brain directly but non-invasively and has been effective in animal models of stroke and Alzheimer's disease. In this study we sought to determine whether intranasal (IN) DFO could be neuroprotective for PD in a rat model. PD was induced with a unilateral injection of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle, while sham surgery rats received saline injections. Rats were pre-treated three times with either IN DFO or saline (starting 4 days before 6-OHDA), and post-treated twice/wk for one month before behavioral tests. In the apomorphine-induced rotational test, IN DFO significantly decreased the number of contralateral turns after injection of apomorphine HCl ($p<0.05$). Also, IN DFO significantly decreased limb asymmetry in the rearing tube as measured with contralateral limb touches ($p<0.05$). The IN DFO treatment yielded a trend towards decreased contralateral foot-slips on the tapered balance beam, though the difference was not significant. Finally, IN DFO-treated rats had increased preservation of tyrosine hydroxylase immunoreactive neurons in the substantia nigra ($p<0.05$). These results confirm that DFO is beneficial in a 6-OHDA model and demonstrate improvement in motor deficits and dopaminergic neuronal survival with non-invasive intranasal delivery, making this an attractive potential treatment for PD.

Abbreviations: DFO, deferoxamine; IN, intranasal; 6-OHDA, 6 hydroxydopamine; PD, Parkinson's disease; AD, Alzheimer's disease; TH IR, tyrosine hydroxylase immunoreactive; PBS, phosphate buffered saline; ICV, intracerebroventricular; LIP, labile iron pool; GSK3β, glycogen synthase kinase 3β; HIF-1α, hypoxia inducible factor-1α; POD, Precision Olfactory delivery; HFA, hydrofluoroalkane; SN, substantia nigra

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1. Introduction

Parkinson's disease (PD) is a neurodegenerative disease that results largely from the death of dopaminergic cells in the substantia nigra (SN), and current treatment options are largely based on compensating for the loss of dopamine in the brain. Another neuropathological trait of PD is a buildup of excess iron in the SN, and thus, metal chelators have been suggested as possible treatment for PD (Youdim et al., 2004; Jellinger, 2013). Deferoxamine (DFO) is an iron chelator that traditionally is used for treatment of iron-overload in human patients, but has also been tested in animal studies to treat PD (Ben-Shachar et al., 1992), Alzheimer's disease (AD) (Percy et al., 2011), and stroke (Selim and Ratan, 2004; Hanson et al., 2009), and can be beneficial when given either before or after disease onset. In a 6-hydroxydopamine (6-OHDA) rat model of PD, systemically delivered DFO alleviated both the build-up of iron in the brain and behavioral problems related to the disease (Youdim et al., 2004; Dexter et al., 2011). Another animal study delivered DFO along with quercetin, a bioflavonoid, via intraperitoneal injections and found similar results (Haleagrahara et al., 2013). While these studies demonstrated that DFO could be used as a treatment for animal models of PD, most used some form of systemic administration, or intracranial injections that are not suitable for human use.

DFO has been used with intranasal (IN) delivery to treat rodent models of neurodegenerative disease and holds potential as a clinically relevant option for the treatment of PD (Hanson and Frey, 2008). Intranasal delivery allows for treatments to be targeted to the brain through application to the nasal cavity, and is beneficial for several reasons. Primarily, IN delivery allows drugs to bypass the blood-brain barrier and target the CNS directly via the olfactory and trigeminal nerves (Hanson and Frey, 2008; Dhuria et al., 2010), while minimizing unwanted systemic side-effects (Pires et al., 2009). Intranasal delivery of drugs has been used in both animals models and humans (Born et al., 2002; Hanson et al., 2009; Marks et al., 2009; Dhuria et al., 2010). Direct brain targeting of DFO via IN administration is especially beneficial for DFO because DFO has a short half-life in blood (Summers et al., 1979; Allain et al., 1987). Intranasal DFO has been tested in mouse and rat models of stroke, AD, and PD (Hanson et al., 2009; Fine et al., 2012; Febbraro et al., 2013). Recently, Febbraro et al. (2013) used an α-synuclein rAAV model of PD and treated awake rats intranasally with DFO. They found that DFO improved behavioral deficits as well as prevented an increase in Fe^{3+} positive cells (Febbraro et al., 2013).

In the current study, we determined whether IN DFO would alleviate behavioral and biochemical symptoms in a 6-OHDA model of PD in rats. The 6-OHDA destroys dopaminergic neurons, thus mimicking PD (Duty and Jenner, 2011). Rats underwent stereotaxic surgery and were given a unilateral injection of either 6-OHDA or saline to the right side of the brain in the medial forebrain bundle. The rats were treated with either IN DFO or PBS using a Precision Olfactory Delivery (POD) device (Impel Neuropharma, Seattle, WA), which targets drugs to the upper third of the nasal cavity using hydrofluoroalkane (HFA) gas. The DFO-treated groups of rats were given IN DFO both before and after administration of 6-OHDA, as disease onset occurs immediately upon administration of 6-OHDA rather than as a progressive neurodegenerative disorder, which is a weakness of the model (Duty and Jenner, 2011). Behavior tests included the apomorphine-induced rotational test, rearing tube, tapered balance beam, and open field test. Rats were then euthanized and brain tissues were examined. We found that IN DFO improved behavior in both the apomorphine-induced rotational test and rearing tube test, and indicated improvement in the balance beam test. Also, the tissue analysis showed that IN DFO protected against the loss of dopaminergic neurons in the lesioned area. These results indicate that IN DFO is a potential method of protecting against and treating Parkinson's disease.

2. Results

2.1. General health

There were no obvious health problems or mortality for any rats during the study. There was no significant difference in

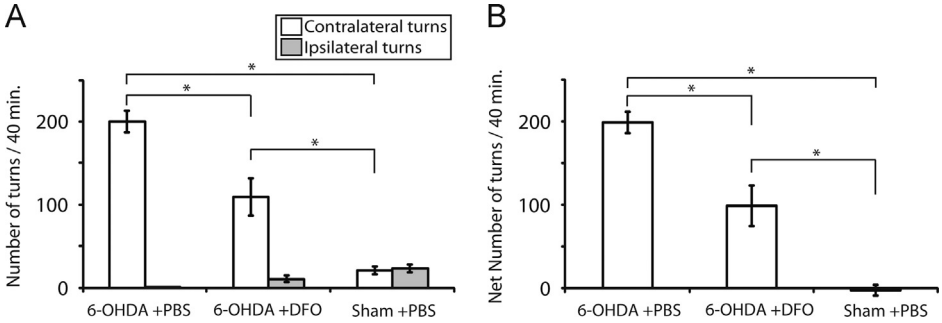

Fig. 1 – Apomorphine-induced rotational test data for (A) number of turns and (B) net number of turns (contralateral–ipsilateral). Long Evans rats were dosed with IN DFO or PBS for 5 weeks (n=6–11 rats/group). Each rat was given a subcutaneuous injection of apomorphine HCl (Sigma-H116) (0.4 mg/kg) and observed for 40 min. 6-OHDA+PBS displayed significantly more total turns than Sham+PBS for both measures (*$p<0.05$). 6-OHDA+DFO had less turns than 6-OHDA+PBS for both measures (*$p<0.05$). Error bars are SEM. 6-OHDA=6 hydroxydopamine, PBS=phosphate-buffered saline, IN=intranasal, DFO=deferoxamine, and SEM=standard error of the mean.

weights between any groups, which included the 6-OHDA+PBS, 6-OHDA+DFO, and Sham+PBS($p>0.05$).

2.2. Apomorphine-induced rotational test

There were no differences in analyses for this test for trials run at 10 days or 5 weeks, so the data were combined. For average number of contralateral turns, 6-OHDA+PBS rats turned 200 times in the 40 min. collection period, which was significantly more than Sham+PBS rats (21 turns) (Fig. 1; $p<0.05$). This deficit was significantly improved in the IN DFO rats, who had only 109 turns ($p<0.05$). The same significant effect was seen in the average difference in net turns (contralateral turns minus ipsilateral turns), in which 6-OHDA+PBS rats had 199 turns, 6-OHDA+DFO rats had 99 turns, and sham PBS rats had −3 turns (Fig. 1; $p<0.05$ between all groups).

2.3. Rearing tube

For average number of contralateral paw touches, 6-OHDA+PBS rats had significantly fewer touches (0.4) than Sham+PBS rats (6) ($p<0.05$). This deficit was significantly improved in 6-OHDA+DFO rats, who averaged 2.6 touches (Fig. 2; $p<0.05$). The same effects were also seen when analyzed as a percentage of contralateral touches out of total contra and ipsilateral touches. Sham+PBS and 6-OHDA+DFO rats both had a significantly higher percentage of contralateral touches (34% and 37% respectively) than 6-OHDA+PBS rats (9%; Fig. 2; $p<0.05$).

2.4. Tapered balance beam

Sham+PBS rats had significantly fewer contralateral footslips (1.7) than 6-OHDA+PBS rats (6.5) ($p<0.05$), showing that this test could detect a change in the 6-OHDA model (Fig. 3). 6-OHDA+DFO rats also had fewer contralateral footslips (5) than 6-OHDA+PBS rats, which suggests a trend towards an effect of DFO-treatment, but the difference was not statistically significant. All groups showed a similar number of ipsilateral footslips.

2.5. Open field

Sham+PBS rats showed a significantly higher average velocity (8.4 cm/s) than 6-OHDA+PBS and 6-OHDA+DFO rats (5.6 and 5.9 cm/s, respectively), demonstrating that there was a detectable change in the 6-OHDA model ($p<0.05$), but no effect of treatment with IN DFO ($p>0.05$) (data not shown). This deficit in the PD model was also seen for total activity, as Sham+PBS rats also had significantly more average line crossings (79) than 6-OHDA+PBS rats (40) and 6-OHDA+DFO (41) rats ($p<0.05$). As with velocity, 6-OHDA+DFO and 6-OHDA+PBS had a similar average number of line crossings with no significant difference for an effect of DFO-treatment ($p<0.05$).

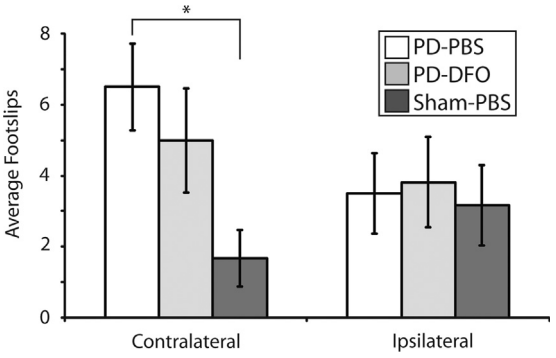

Fig. 3 – Limb coordination as measured by average footslips in tapered balance beam. Rats were dosed with IN DFO or PBS for 5 weeks ($n=6$–11 rats/group). Sham+PBS rats had significantly fewer contralateral footslips than 6-OHDA+PBS rats (*$p<0.05$) demonstrating an effect of the 6-OHDA model in this test. 6-OHDA+DFO rats also had fewer contralateral footslips than 6-OHDA+PBS rats though the difference is not significant. There were no differences in ipsilateral footslips for any group. Error bars are SEM. 6-OHDA=6 hydroxydopamine, IN=intranasal, DFO=deferoxamine, PBS=phosphate-buffered saline, and SEM=standard error of the mean.

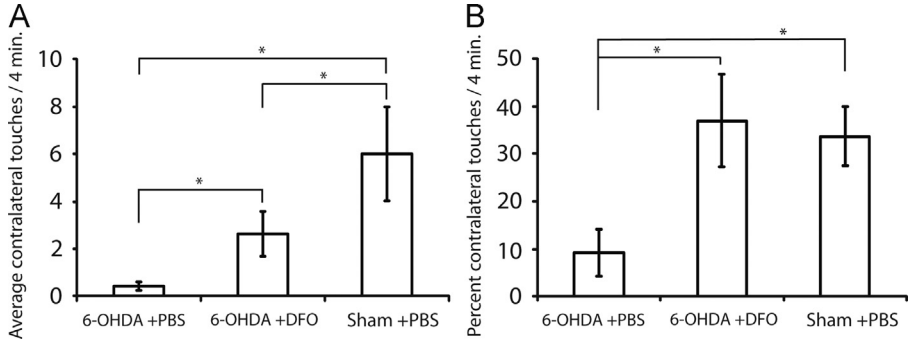

Fig. 2 – Forelimb use asymmetry as measured by rearing tube, as average contralateral touches (A) and percent contralateral touches (B). The number of paw touches against a clear cylinder (25 cm × 40 cm) was observed after 4 min. of activity. Rats were dosed with IN DFO or PBS for 5 weeks ($n=6$–11 rats/group). Sham+PBS rats had significantly more contralateral touches than 6-OHDA+PBS rats (*$p<0.05$), while 6-OHDA+DFO rats also had significantly more contralateral touches than 6-OHDA+PBS (*$p<0.05$). Error bars are SEM. 6-OHDA=6 hydroxydopamine, IN=intranasal, DFO=deferoxamine, PBS=phosphate-buffered saline, and SEM=standard of the mean.

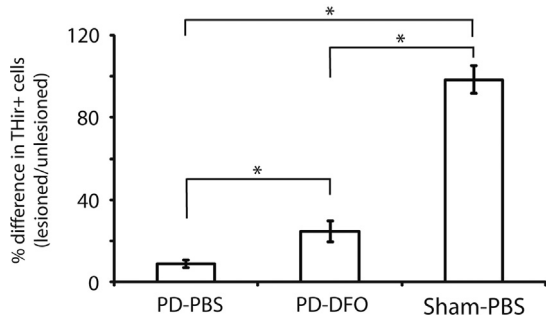

Fig. 4 – Percent difference in tyrosine hydroxylase immunoreactive cells after unilateral injection of 6-OHDA. Rats were dosed with IN DFO or PBS for 5 weeks. After behavior tests, images of the TH-IR cells in the lesioned and unlesioned sides of the brain were captured, and the percent difference of the lesioned to unlesioned side determined. 6-OHDA+PBS rats had a significant decrease of TH-IR cells on the lesioned side compared to Sham+PBS (*$p<0.05$). The percent difference between PD-PBD and 6-OHDA+DFO was also significant (*$p<0.05$). Error bars are SEM. TH-IR=tyrosine hydroxylase immunoreactive, IN=intranasal, DFO=deferoxamine, PBS=phosphate-buffered saline, 6-OHDA=6 hydroxydopamine, and SEM=standard error of the mean.

2.6. *Tyrosine Hydroxylase Staining*

Delivery of 6-OHDA into the medial forebrain bundle significantly reduced TH-IR (tyrosine hydroxylase immunoreactive) cells on the lesioned side of the brain compared to the unlesioned side (percent difference; Figs. 4 and 5). Sham+PBS rats showed a significantly higher average in percent difference in TH-IR cells (98.49%) than 6-OHDA+PBS rats (8.71%; $p<0.05$). The ~90% loss of TH-IR cells on the lesioned side closely approximates what would be expected for a unilateral dose of 10 μg of 6-OHDA (Duty and Jenner, 2011). A significant effect of IN DFO treatment was demonstrated, as 6-OHDA+DFO rats exhibited a percent difference in TH-IR cells (24.5%) greater than that of 6-OHDA+PBS rats ($p<0.05$).

3. Discussion

This study demonstrates a neuroprotective effect of intranasally delivered DFO in a 6-OHDA model of PD. Treatment with IN DFO significantly improved performance in the apomorphine-induced rotational test and in the rearing tube, with a trend of improvement on the balance beam. IN DFO also significantly decreased the loss of dopaminergic neurons in the lesioned side of the brain. Intranasal DFO was safe and well tolerated as evidenced by comparable weight gain, lack of overt behavioral abnormalities, and no mortality. Previous studies have shown that DFO protects against the 6-OHDA induced dopaminergic neurodegeneration when delivered ICV (Youdim et al., 2004) and IP (Dexter et al., 2011; Haleagrahara et al., 2013), and this study shows that IN is also a viable alternative for delivery. This study also supports Febbraro et al. (2013), who show that IN DFO is beneficial in a transgenic PD rat model of α-synuclein accumulation. Intranasal delivery is noninvasive and has practical clinical applications. In comparison to systemic delivery, IN delivery is targeted directly to the brain and may lead to fewer side effects, which have been problematic in previous clinical studies with systemic DFO administration (Crapper McLachlan et al., 1991).

To the best of our knowledge, this is the first study to use an intranasal spray device in a rat model of Parkinson's disease. Use of the POD device provides more efficient intranasal drug delivery as it targets drugs to the upper third of the rat nasal cavity (Hoekman and Ho, 2011). This ensures that the majority of drug makes contact with the olfactory and trigeminal nerve pathways, making it more likely that the drug reaches the brain. Additionally, use of an IN spray device decreases the time spent under anesthesia, which is beneficial both for animal safety and because exposure to anesthesia may be either neuroprotective or neurotoxic, depending on the circumstances (Karmarkar et al., 2010). Rats in this study spent approximately 4–5 min under anesthesia for each day of dosing, whereas rats receiving IN drops with a pipettor can be under anesthesia for 40 min to an hour (Hanson et al., 2009). Similar intranasal spray devices (including attachments to Impel's POD device) are available for IN delivery to primates and humans, which could ease the transition from animal studies to clinical trials.

Intranasal DFO decreased Parkinsonian behavior. In all four behavioral tests, there was a significant difference between 6-OHDA+PBS and Sham+PBS rats, demonstrating the successful creation and validity of the 6-OHDA Parkinson's rat model. Intranasal DFO treatment significantly decreased rotational behavior in the apomorphine-induced rotational test and significantly decreased forelimb asymmetry in the rearing tube test, supporting our biochemical findings that DFO protected dopaminergic neurons. Although it seemed odd that the PBS-treated sham rats had 34% contralateral touches (instead of an expected 50%), which may be due to natural variability from the small sample size or a natural handedness in the rat population (Guven et al., 2003), it seems most relevant that it is still significantly higher than the 9% contralateral touches by the 6-OHDA rats. We also used the tapered balance beam test to evaluate skilled walking and balance, with increased foot-slips indicating impairment, which was also used in Madete et al. (2011). Although the DFO treatment decreased average foot-slips, this change did not reach significance. It is not known how or why the DFO mitigated toxicity to 6-OHDA in some behavior tests but not others, but may be related to the mechanism of action. In future studies, we plan to increase the sensitivity of our testing in balance and skilled walking by using two additional tests, the narrow balance beam and horizontal ladder test, which could be beneficial in this model (Allbutt and Henderson, 2007; Metz and Whishaw, 2009).

Treatment with IN DFO reduced the loss of dopaminergic neurons as measured by tyrosine hydroxylase immunoreactive neurons. Since tyrosine hydroxylase is the rate-limiting step in the biosynthesis of dopamine, it indirectly estimates the ability of the brain to synthesize dopamine. In comparison to the sham rats, the PD rats had about a 90% loss of TH neurons on the lesioned side, which is consistent with a 'full'

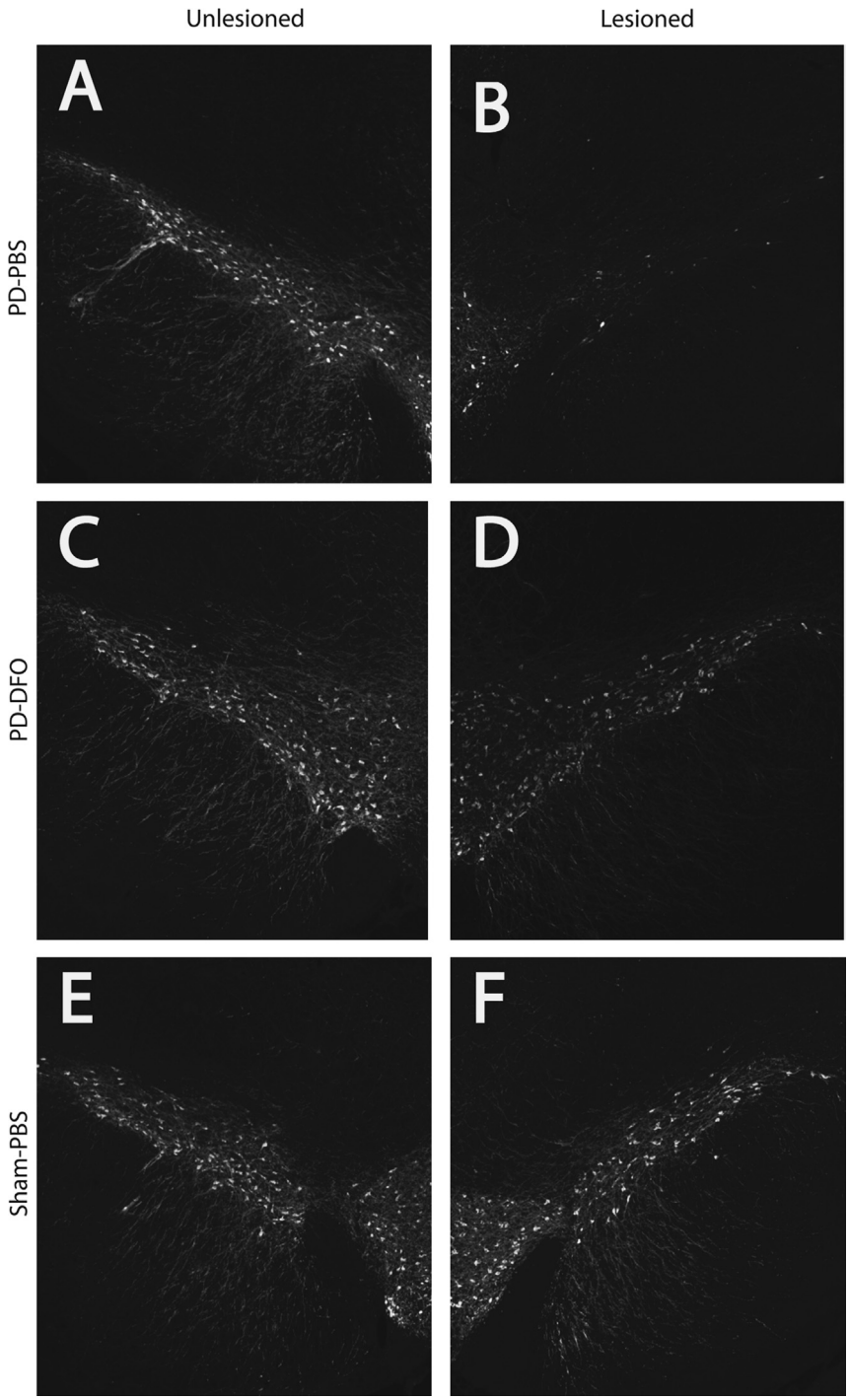

Fig. 5 – Representative photomicrographs of TH-IR cells from unlesioned and lesioned tissue from 6-OHDA+PBS (A and B), 6-OHDA+DFO (C and D), and Sham+PBS (E and F). Images are at 40 × magnification. Images qualitatively depict the reduction of TH-IR cells in the lesioned section of 6-OHDA+PBS as evidenced by stained cells. TH-IR=tyrosine hydroxylase immunoreactive, IN=intranasal, DFO=deferoxamine, PBS=phosphate-buffered saline, and 6-OHDA=6 hydroxydopamine.

lesion for the dose of 6-OHDA given, and leads to the behavioral deficits seen in the apomorphine-induced rotational test and rearing tube (Duty and Jenner, 2011). The rats from the IN DFO group had a significantly lower percent difference in TH-IR cells as compared to the 6-OHDA+PBS group, suggesting that the IN DFO protected against the toxicity caused by 6-OHDA. Other studies have shown similar results when using systemic DFO in 6-OHDA rats (Youdim et al., 2004; Dexter et al., 2011). It is not known whether the timing of treatment with DFO, which occurred before, during, and after lesioning with 6-OHDA, played a role in mitigating damage to dopaminergic neurons. In a rat stroke model, IN DFO decreased infarct volume when delivered either before or after induction of the model (Hanson et al., 2009). The question of the window of opportunity for delivery of DFO will be addressed in future studies.

There are several possibilities for how DFO may be beneficial in a 6-OHDA model. The beneficial effects of DFO may stem from a general neuroprotective mechanism, as evidenced by the fact that DFO is beneficial in a number of different diseases, including animal models of stroke, both amyloid and tau models of AD, PD, traumatic brain injury, subarachnoid hemorrhage, and more (Hanson et al., 2009; Dexter et al., 2011; Lee et al., 2011; Fine et al., 2012; Hanson et al., 2012; Zhang et al., 2013). One commonality of these neurodegenerative diseases is the imbalance in iron regulation. One possible mechanism by which DFO is protective for PD is by chelating iron from the labile iron pool (LIP), thereby decreasing free radical damage via Fenton chemistry, as suggested by others (Youdim et al., 2004; Dexter et al., 2011; Febbraro et al., 2013). Indeed, 6-OHDA releases excess iron bound in ferritin into the LIP causing increased oxidative damage (Oestreicher et al., 1994; Mazzio et al., 2004), and DFO may act by sequestering this reactive iron. Similarly, the destruction of dopaminergic neurons also releases sequestered iron from neuromelanin, which can also increase ferric iron in the LIP. Dexter et al. (2011) were able to quantify a reduction in hydroxyl radical formation with DFO treatment. Another possible mechanism of DFO includes regulation of glycogen synthase kinase 3β (GSK3β), as IN DFO has been shown to activate the phosphorylation of GSK3β in brain tissue (Fine et al., 2012). Elevated levels of GSK3β have been shown in post-mortem brain tissue analyses from human PD patients and mouse models of PD (Lei et al., 2011). Another mechanism may be related to hypoxia inducible factor-1α (HIF-1α), which is commonly known to be regulated by DFO, and recently shown to be regulated *in vitro* in the brain using IN delivery in mice (Fine et al., 2012). HIF-1α is a transcription factor that up-regulates a number of target genes, many of which may play a role in neuroprotection, some of which may be important for PD (Weinreb et al., 2013).

Intranasal DFO treatments proved to be beneficial in the 6-OHDA rat model of PD in respect to both behavioral and biochemical effects. Rats treated with IN DFO performed significantly better in two of the four behavior tests completed in this study. They also retained significantly more dopaminergic neurons in the lesioned area of their brain as compared to the group treated with saline. These results suggest that DFO, which has been used successfully as a treatment in humans for decades, could be a potential treatment option for PD. Future studies will examine the window of opportunity for treatment of the 6-OHDA model of PD, and could possibly involve the use of a partial lesion model. Ideally, these studies will lead to the use of IN DFO in a clinical study of PD, and advance the treatment of PD.

4. Experimental procedures

4.1. Experimental design

Rats were acclimated to handling over the course of two weeks, and then divided into three groups that underwent: (1) stereotaxic injection of 6-OHDA (Sigma; H116; St. Louis, MO) and treatment with IN PBS (6-OHDA+PBS; $n=11$), (2) stereotaxic injection of 6-OHDA and IN treatment with DFO (6-OHDA+DFO; $n=11$), and (3) Sham surgery (stereotaxic injection of saline) and treatment with IN PBS (Sham+PBS; $n=6$). Before surgery, rats were pre-treated intranasally as described below, and after surgery they were treated twice/wk for four wk. Behavior tests began the fifth week after surgery, and dosing continued during the week of behavior tests. After behavior tests, the rats were euthanized and brain tissue collected for analysis of (TH) immunoreactive cells.

4.2. Animal care and treatment

A total of 28 male Long Evans rats were purchased from Harlan Laboratories (Indianapolis, IN). The rats were 3 months old at time of purchase (Mean weight=409.5 g, St Dev=45.1 g). Rats were housed separately and allowed free access to water and food during a 12-h light/dark cycle. Behavioral testing and dosing were performed during the day portion of the circadian cycle. All procedures were approved by the Institutional Animal Care and Use Committee of HealthPartners Institute for Education and Research at Regions Hospital under protocol 11-110.

4.3. 6-OHDA model

Rats were anesthetized with isoflurane. Their heads were shaved and IP injections of 20 mg/kg desipramine HCl (Sigma; D3900) administered at 30 min prior to delivery of 6-OHDA to prevent damage to noradrenergic neurons as in Breese and Traylor (1972), and Tessel et al. (1978). Rats were positioned in a stereotaxic frame with a nose-cone for continuous administration of isoflurane. A midline incision was made from the mid-orbital region to the occipital ridge. A hole was drilled through the skull at A/P: −3.8, M/L +1.6 (on the right side). The 6-OHDA was prepared with saline (2.5 mg/mL). Then, a 10 μL Hamilton syringe was filled with freshly prepared 6-OHDA (10 μg per rat) and lowered 8.3 mm (from dura mater) into the brain. The 6-OHDA or saline was delivered at a rate of 1 μL/min for 4 min. The needle was left in place for 4 min. before removal to ensure complete delivery. The skull was sealed with bone wax (Covidien, Mansfield, MA) and the skin sutured closed. The coordinates were chosen to target the median forebrain bundle based on Paxinos and Watson (1986).

4.4. Drug administration

Deferoxamine mesylate salt was purchased from Sigma (D9533) and was delivered as a 10% solution in 0.2× PBS (Sigma; P4493) at a pH of 6.0. Total dose was 3 mg DFO/administration. Rats were pre-treated 4 d, 2 d, and 30 min prior to surgery with either DFO or 0.2× PBS. Post-surgery, rats were treated twice weekly for four wk. For each session of drug administration, rats were anesthetized with isoflurane gas. The rat was placed on its back, and the POD nasal device (Impel Neuropharma, Seattle, WA) was inserted 14 mm into the right nostril. PBS or DFO (15 μL) was delivered to the right nostril. The process was then repeated for the left nostril after 2 min. The total time to dose a rat for each administration was 4–5 min.

4.5. Behavior assessments

Beginning 33 days after surgery, rats were subjected to a 5-day battery of behavior tests to measure motor function. The battery consisted of 2 days of balance beam, and 1 day each of open field, rearing tube, and the apomorphine-induced rotational test.

4.5.1. Apomorphine-induced rotational test

This test was used to measure motor asymmetry caused by the unilateral injection of 6-OHDA, and is common for the 6-OHDA model (Rizelio et al., 2010; Duty and Jenner, 2011). The test was conducted twice; 10 d and 5 wk postoperatively. Rats with a unilateral injection of 6-OHDA are expected to turn contralateral to the lesion when given R-(-)-apomorphine. Rats were subcutaneously injected with 0.4 mg/kg apomorphine HCl (Sigma; A4393), and immediately placed in a circular bucket, undisturbed for 40 min, while activity was recorded on video. Between each rat, the buckets were cleaned with disinfectant wipes and paper towels. The number of 360° contralateral and ipsilateral turns completed in the 40 min after injection was manually quantified from the video by a blinded observer.

4.5.2. Rearing tube

Forelimb use asymmetry was measured by placing each rat in a clear cylinder (25 cm × 40 cm) for four min, and recording its activity on video. The video camera was placed in a fixed location in front of the cylinder, and a large mirror was placed behind to allow a clear viewing all around the cylinder. The number of times a rat touched the side of the cylinder with its contralateral paw, ipsilateral paw, and both paws simultaneously while rearing was manually quantified by a blinded observer. The video was filmed in high definition and watched in slow motion to allow easy detection of paw touches. The method was based on those in Falk et al. (2011) and Tillerson et al. (2001).

4.5.3. Tapered balance beam

Balance was tested by counting the number of footslips as each rat crossed a tapered balance beam. The beam was 1.34 m long, elevated 0.91 m off the ground, and decreased in width from the starting point (6.5 cm) to the end point (1.9 cm). Each rat was placed on a platform at the wide end of the beam, and bright lights and fans provided an incentive for the rat to reach an escape box at the narrow end. The beam had a raised center path for walking, and a lower ledge on each side. Blinded observers counted the number of footslips on each side of the beam (a footslip occurs when any of the rat's feet slip from the raised walking path and contact the lower ledge of the beam). This test consisted of a training day (during which each rat became accustomed to the beam and learned to cross it), followed the next day by a testing day (during which each rat performed 3 consecutive trials, spending 20 s. in the escape box between each trial). Footslips were recorded on testing day only, and total footslips for the three trials were combined for analysis. This test is similar to the narrow balance beam test often used in Parkinson's models (Allbutt and Henderson, 2007) but potentially decreases compensatory behavior, thereby revealing deficits more clearly (Woodlee and Schallert, 2004). The method used here is similar to that used to test motor function in models of stroke and spinal cord injury (Biernaskie et al., 2004; Spanevello et al., 2013), and a variation on the tapered balance beam has also been used in the 6-OHDA Parkinson's model (Madete et al., 2011).

4.5.4. Open field

Locomotor activity was assessed with an open field test. The open field was a white rectangular open-top box measuring 85 cm (length) × 77 cm (width) × 44 cm (height). Each rat was placed in the center of the box and allowed to explore for 4 min. Activity was recorded with an overhead video camera. Using the EthoVision tracking system (Version 3.1; Noldus, Leesburg, VA, USA), the open field box was divided by a virtual grid with 16 equal sized boxes and the total number of times the rat crossed one of these imaginary lines (line crossings) was recorded as a measure of activity. The rat's average velocity was also calculated by the EthoVision tracking system. This method was based on those in Brown et al. (2011) and Rizelio et al. (2010), who measured changes in locomotor activity in the 6-OHDA rat model.

4.6. Euthanasia and tissue collection

Rats were anesthetized with a cocktail of ketamine HCl (30 mg/kg), xylazine HCl (6 mg/kg), and acepromazine (1 mg/kg) and then transcardially perfused with 0.9% saline (60 mL) followed by paraformaldehyde (110 mL). The brain was removed and placed upside down in a coronal brain matrix. An initial slice was made where the anterior optic nerves join at the optic chiasm. From there, 6 slices were made at 2 mm each, one anterior to the initial cut and five posterior. The slices were post-fixed in paraformaldehyde for 24 h, then transferred to a 20% sucrose solution until analyzed.

4.7. Tissue analysis

Tissue from 20% sucrose solution was frozen in Tissue-Tec Optimal Cutting Temperature Compound (Sakura Finetek, Torrance, CA). Three 20 μm crysosectioned coronal sections, spaced between −5.0 and −6.0 Bregma, were collected from each rat using a Leica CM3050 cryostat. The tissue was blocked for one hr using serum blocking solution (2% goat serum, 1% BSA, 0.05% Tween 20, 0.05% sodium azide, 1 × PBS) and incubated with a 1:300 dilution of anti-tyrosine hydroxylase (Millipore AB 152, Billerica, MA) in 1 × PBS overnight at 4 °C. Sections were washed and incubated with a 1:200 dilution of Alexa Fluor 488 Goat anti-rabbit IgG (Invitrogen A11008, Grand Island, NY) for one hour at room temperature. Tissue was mounted in Prolong Gold Anti-fade Reagent with DAPI (Life Technologies, Grand Island, NY). Fluorescent images of the substantia nigra were captured with a Nikon A1 Spectral Confocal Microscope at a 2048 × 2048 pixel resolution (40 × magnification). Tyrosine hydroxylase positive neurons were automatically counted within a specified ROI outlining the substantia nigra using NIS Elements AR Analysis software (the ROI was selected by a blinded observer). Data was presented as percentage of TH+ neurons in the lesioned side relative to the intact side (percent difference).

4.8. *Data analysis*

Data were analyzed with ANOVA with Fishers least significant determinant for post-hoc comparisons. Comparisons were made between Sham+PBS and 6-OHDA+PBS to demonstrate a deficit in this rat model of Parkinson's, and between 6-OHDA+DFO and 6-OHDA+PBS to determine whether the IN DFO treatment was beneficial. For the apomorphine-induced rotational test and rearing tube, rats were excluded from analysis using Grubb's test for outliers (GraphPad Prism software; La Jolla, CA). For rearing tube, rats were also excluded from analysis if they did not rear at all. Up to 1 rat/group was removed from analysis for these two tests, which affected sample size for analysis.

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