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Chronic intranasal deferoxamine ameliorates motor defects and pathology in the α-synuclein rAAV Parkinson's model

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ABSTRACT

Parkinson's disease is characterized by neuronal death in the substantia nigra and the presence of intracellular inclusions of α-synuclein in the Lewy bodies. Several lines of data support a role for iron in Parkinson's disease: iron is present in Lewy bodies, iron accumulates in the dopaminergic neurons in the substantia nigra, and Parkinson's disease is correlated with polymorphisms of several genes implicated in iron metabolism. Furthermore, iron can compromise the solubility of α-synuclein through direct interaction and can induce neurotoxicity *in vitro*. Here, we investigate the possible neuroprotective effect of the iron chelator deferoxamine *in vivo* to elucidate whether iron chelation can provide meaningful therapy for Parkinson's disease. Hence, we used a Parkinson's disease animal model based on unilateral injection of a recombinant adeno-associated viral vector encoding α-synuclein in the rat midbrain. Rats were treated with a novel deferoxamine delivery approach: 6 mg of the compound was administered intranasally three times a week for 3 or 7 weeks. The behavior of the animals and histopathological changes in the brain were analyzed. Our data show that although intranasal administration of deferoxamine in rats did not protect them from dopaminergic cell death, it did decrease the number of the pathological α-synuclein formations at the terminal level. In addition, this treatment resulted in changes in the immune response and an overall partial improvement in motor behavior. Taken together, our data show that *in vivo* iron chelation can modulate α-synuclein-induced pathology in the central nervous system. Our data suggest that chronic administration of intranasal deferoxamine may be a valid approach to limiting the mishandling of α-synuclein in the central nervous system observed in Parkinson's disease and slowing disease progression.

Introduction

Parkinson's disease is mainly characterized by dopaminergic cell death in the substantia nigra (SN) and the presence of characteristic intracytoplasmatic inclusions, called Lewy bodies (LBs), in the surviving neurons (Marti et al., 2003). LBs are primarily composed of fibrillar aggregates of α-syn but also contain other molecules, such as iron (Castellani et al., 2000; Spillantini et al., 1997). In addition to being the main component of LBs, the role of α-syn in the disease is further supported by the fact that point mutations or multiplication of the gene results in familial PD (Ross et al., 2008; Thomas and Beal, 2007). Several groups have shown that, similar to α-syn, iron homeostasis is altered in PD, and both the protein and the metal accumulate in brains affected by PD or other synucleinopathies (Perry and Yong, 1986; Youdim et al., 1989; Zecca et al., 2004). The correlations between polymorphisms of different genes involved in iron homeostasis (Transferrin, Transferrin receptor, Frataxin, Lactoferrin and Haemo-chromatosis-related protein gene) and the incidence of sporadic cases of PD also support a role for this metal in the neurodegenerative process (Borie et al., 2002; Oakley et al., 2007). Indeed, several findings corroborate a role for iron in the progression of PD neurodegeneration (Double et al., 2000; Gerlach et al., 1994). Accordingly, overexpression of ferritin or the use of iron chelators results in neuroprotection in toxic PD models, although this has never been tested *in vivo* α-syn-based PD models (Kaur et al., 2003; Matarredona et al., 1997; Shachar et al., 2004; Xu et al., 2008, 2010).

Iron accumulation has recently been proposed as an early marker of PD, suggesting that changes in iron handling are pathogenic events that could play causative roles in the disorder (Martin et al., 2008). However, whether increases of iron in the SN are a casual factor or a consequence of the disease is still debated, especially because the mechanisms of iron's interference with brain homeostasis are not completely understood (Berg et al., 2001; Thompson et al., 2001). It has been shown *in vitro* that ferrous iron (Fe^{2+}) promotes oxidative stress (Beal, 1992; Gutteridge, 1992), which may contribute to the oxidative damage found in PD and might indirectly influence other

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proteins, such as α-syn. Further supporting this idea, *in vitro* studies have shown that iron can bind to α-syn and promote its aggregation by inducing α-syn oxidation (Cole et al., 2005; Golts et al., 2002; Gotz et al., 2004; Hashimoto et al., 1999). Additionally, recent data have revealed a possible direct link between the presence of iron and α-syn expression based on the existence of a putative iron responsive element in the 5′ untranslated region of human α-syn mRNA (Friedlich et al., 2007). Accordingly, we have shown *in vitro* that α-syn expression is modulated by iron at the translational level (Febbraro et al., 2012). Furthermore, it has recently been proposed that α-syn is a ferrireductase that can reduce Fe^{3+} to Fe^{2+} (Davies et al., 2011). Although a direct correlation between iron and α-syn in PD has not yet been fully proven, it is clear that both the protein and the metal are crucial for correct brain function.

Due to the role of iron in oxidative stress, iron chelators have been used as a neuroprotective strategy in different *in vitro* and *in vivo* neurotoxic models of PD. Deferoxamine (DFO), one such chelator, acts by binding Fe^{3+} and thereby preventing iron ions from catalyzing redox reactions that lead to free radical formation (Cullen et al., 2009). DFO was first used for the treatment of disorders related to iron overload and has been shown to protect neurons in the 6-OHDA and MPTP animal models of PD (Ben-Shachar et al., 1992; Kaur et al., 2003; Shachar et al., 2004). In the present study, we use a recently validated intranasal drug delivery approach to administer DFO more efficiently to the CNS (Hanson et al., 2009) in an animal model of PD based on the overexpression of α-syn using recombinant adeno-associated viral (rAAV) vectors. Our data show that although DFO treatment does not result in significant protection against nigral dopaminergic cell death in the α-syn overexpressing animals, it has an effect on the number of α-syn species that accumulate pathologically and the microglia response. These effects resulted in partial protection from the motor defects induced by α-syn overexpression. Taken together, our findings support a role for iron in α-syn-induced neuropathology and suggest that iron chelation may be meaningful in the treatment of PD.

Materials and methods

Recombinant adeno-associated viral vector production

The rAAV2/5 vector produced contained the coding sequence for the wild-type human α-syn under the control of a chimeric promoter consisting of an enhancer element from cytomegalovirus followed by the chicken β-actin promoter and flanked by AAV2 Inverted Terminal Repeats (Xu et al., 2001). The vector was produced by co-transfection of HEK293 cells with a pTR-UF20 plasmid containing the human α-syn gene with a helper plasmid containing the necessary adenoviral packing genes. The rAAV2/5 vector (rAAV2 vector packaged in AAV5 capside) was purified by iodixanol step gradients and ion-exchange chromatography as described previously (Zolotukhin et al., 1999, 2002), and vector titer was determined by quantitative PCR. The final titer for the vector was 8.0×10^{12} genome copies/ml.

Animals, surgery and intranasal drug delivery

Adult female Sprague Dawley rats (n = 64) from Taconic, Denmark, were used for the experiments. Animals weighed 225–250 g at the beginning of the experiment. They were housed three in a cage with *ad libitum* access to food and water and exposed to a 12 h light/dark cycle according to regulations of the Faculty of Health Sciences, Aarhus University, which adhere to Danish and EU law. All experimental protocols were previously approved by the Danish Animal Experiments Inspectorate.

For surgery, rats were anesthetized with a mix of dormitor-fentanyl (40 mg/kg body weight, i.p.). The surgical procedure was carried out using a stereotactic frame (Stoelting, Wood Dale, IL, USA). The rat scalp was incised along the midline to expose the bregma. Next injection coordinates for viral vector delivery were calculated. The coordinates were the following: anteroposterior −5.5 mm; lateral, −2.0 mm; ventral, −7.2 mm to dura, (nose −3.3). Two microliters of viral vector suspension were released unilaterally into the SN of the brain with a Hamilton syringe fitted with a glass capillary (outer diameter 60–80 μm) needle at the rate of 0.2 μl/30 s. The needle was left in position for an additional 5 min before being slowly retracted. Animals were then sutured with metal clips and, when fully awake, returned to their cage where food and water were freely available. For histological analysis, animals were sacrificed at 4 (n = 32, 16 in the DFO group and 16 in the control group) or 8 weeks (n = 32, 16 in the DFO group and 16 in the control group) from the time of injection.

Rats (n = 32, each group) were treated intranasally with either 10% DFO (6 mg per dose, Sigma) or saline solution (vehicle, for the control group) that was adjusted to a pH of 4.65. The solutions were administered as five doses of 12 μl solution (6 μl for each nostril) for a total volume of 60 μl. There was a 2 min gap between each of the five doses. The administration was performed in awake rats and required two persons; one person held the animal lightly with the nostrils exposed, and the second person placed the drops in the nostrils using a pipette. The rats were treated 3 times a week for a period of either 3 or 7 weeks starting one week post-surgery.

Behavioral analysis

Stepping test

During the 7th post-surgery week, animals were tested for forelimb akinesia using a stepping test that has been described previously (Olsson et al., 1995). On the two days prior to the test, the experimenter handled the animals to familiarize them with the test procedure. The test was performed two times per day for 3 consecutive days. The experimenter, blinded to the animal's treatment, held the animal, immobilized the hind limbs with one hand, immobilized the forepaw that was not tested with the other hand, and allowed the free forelimb to touch the table. The number of adjusting steps was counted while the rat was moved sideways along the table surface (90 cm in 5 s) in both forehand (*i.e.*, the animal was pulled to the left when the right paw was unrestrained) and backhand directions. As suggested in previous papers (Kirik et al., 1998), the backhand stepping scores were used as a control for animal performance, and the measurements of the forehand steps were used to evaluate motor impairment. The mean data obtained on the three days constituted the dependent variable.

Cylinder test

The cylinder test is a drug free test that is used to quantify forelimb use in animal models of PD (Schallert et al., 2000). The animals were tested at 4 and 8 weeks post-surgery after being placed in a 20 cm wide clear glass cylinder in which they could move freely. The experiment was videotaped. Mirrors were placed behind the cylinder to ensure all paw placements around the cylinder were visualized. An observer blinded to the animal's treatment viewed the videotapes and counted a minimum of 20 forelimb contacts with the cylinder wall. The number of contralateral (left) forelimb contacts, expressed as a percentage of total contacts, was the actual dependent variable.

Drug-induced rotation tests

Drug-induced rotation tests were assessed 8 weeks post-surgery in automated rotometer bowls after injection of D-amphetamine sulfate (2.5 mg/kg, i.p., Fagron, The Netherlands). Performance was monitored for 90 min. Data (net turns per minute) were analyzed by an experimenter blinded to the animal's treatment.

Histology

Perfusion and tissue processing

At 4 or 8 weeks post-surgery, animals from each group were deeply anesthetized with pentobarbital and perfused through the ascending