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α-synuclein aggregation reduces nigral myocyte enhancer Factor-2D in idiopathic and experimental Parkinson's disease

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ABSTRACT

α-synuclein is an abundant neuronal protein that has been linked to both normal synaptic function and neurodegenerative disease, in particular, Parkinson's disease (PD). Evidence from both *in vitro* and *in vivo* studies indicate that increased wild type or mutant α-synuclein can cause PD, but the molecular mechanisms that underlie α-synuclein-mediated neurotoxicity remain poorly understood. We reported here that myocyte enhancer factor 2D (MEF2D), a nuclear transcription factor known to promote neuronal survival, is down regulated in response α-synuclein accumulation and aggregation. Our data demonstrated that levels of cytoplasmic and nuclear MEF2D were significantly decreased in PD nigral neurons when compared to the nigra of age-matched controls and Alzheimer's disease (AD) cases. This decrease was significantly greater in the nigral neurons with α-synuclein inclusions. Viral vector-mediated overexpression of human α-synuclein in rats resulted in significantly decreased MEF2D in nigral neurons similar to what was seen in PD. The decline of MEF2D-immunoreactivity was associated with a reduction in TH-immunoreactivity. These results indicate that the neuronal survival factor MEF2D is decreased in human and experimental PD, and this decrease is specifically associated with α-synuclein accumulation and aggregation.

Introduction

The transcription factor myocyte enhancer factor-2 (MEF2) is highly expressed in brain, and it regulates gene expression involved in neuronal differentiation, survival, and plasticity (Lin et al., 1996; Heidenreich and Linseman 2004; Mao et al., 1999). Four MEF2 family members (MEF2A, MEF2B, MEF2C, and MEF2D), each encoded by a different gene, have been identified in mammalian cells (Black and Olson, 1998). Recently, MEF2 transcription factors were implicated in synapse formation and maintenance (Flavell et al., 2006, 2008). Knockdown of MEF2A in developing cerebellar granule neurons in organotypic cultures or *in vivo* decreases the formation of synaptic structures (Shalizi et al., 2006). A study in a rodent model of PD revealed that MPTP can induce phosphorylation of MEF2D, resulting in the inactivation of the transcription factor (Smith et al., 2006). The increased phosphorylation of MEF2A and MEF2D was followed by decreased DNA binding, reduced transcriptional activity, and caspase-dependent cleavage to fragments containing N-terminal DNA binding domains and C-terminal transactivation domains (Li et al., 2001). A mutation in the phosphorylation site of MEF2D provides neuroprotection in the MPTP mouse model of PD (Smith et al., 2006). Inhibition of chaperone-mediated autophagy also causes accumulation of inactive MEF2D in the cytoplasm. Mutant α-synuclein inhibited MEF2D function and caused loss of neuronal viability (Yang et al., 2009). These data indicate that inactivation of MEF2D can play a critical role in dopaminergic (DA) neurodegeneration.

Compelling evidence suggested that overexpression of α-synuclein is toxic to nigral neurons in PD (Masliah et al., 2000; Giasson et al., 2002; Shimura et al., 2001; Recchia et al., 2004). The most compelling evidence, perhaps, is the fact that duplication and triplication of the α-synuclein gene alone is capable of causing PD (Singleton et al., 2003; Nishioka et al., 2006). We recently demonstrated that, in PD, α-synuclein aggregation in remaining nigral neuron cause a robust down-regulation of DA markers while adjacent neurons devoid of such aggregates display normal levels of these markers (Chu et al., 2006). A loss of DA phenotype is one of the first degenerative events that occur in vulnerable nigral neurons. Formation of α-synuclein aggregation may be the trigger for this early pathological cascade (Chu and Kordower, 2010). Animal studies support the concept that overexpression of α-synuclein can cause nigrostriatal dysfunction. In *Drosophila melanogaster*, α-synuclein overexpression results in both the degeneration of DA neurons and fly-specific motor deficits (Feany and Bender, 2000). While frank neuronal loss is rarely seen in transgenic mice that overexpress α-synuclein (Masliah et al., 2000; Giasson et al., 2002), these mutants can display a loss of striatal dopamine and a progressive motor syndrome that models specific aspects of the motor deficits seen in PD. More convincing are the gene delivery studies in which viral vectors are used to overexpress α-synuclein within nigral neurons to

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induce a progressive motor dysfunction that is associated with degeneration of the nigrostriatal system in rats and nonhuman primates (Maingay et al., 2005; Kirik et al., 2002; 2003).

Although α-synuclein accumulation and aggregation has been established in PD (Braak et al., 2003; Spillantini et al., 1997; Goedert 2001; Shimura et al., 2001; Nishioka et al., 2006), the mechanism by which DA neurodegeneration occurs as a result of α-synuclein is unclear. In this regard, we investigated the relationship of MEF2D expression with α-synuclein accumulation and aggregation in PD and rats receiving viral overexpression of α-synuclein. The purpose of this study was to determine (1) whether MEF2D immunoreactivity is altered in the remaining nigral neuromelanin (NM)-laden neurons in PD; (2) whether alterations in MEF2D is associated with diminished DA function; and (3) whether alterations in MEF2D in PD is associated with α-synuclein accumulation. To confirm any of these changes in the human brain, we overexpressed α-synuclein in the rodent substantia nigra (SN) and performed similar assessments in this rodent model of PD. To accomplish these aims, the relative level of MEF2D protein was analyzed by using quantitative immunofluorescence intensity measurements within the SN of PD cases. These data were compared with findings seen in age-matched controls as well as Alzheimer's disease (AD) cases.

Materials and methods

Human tissue acquisition and processing

Brains were obtained at autopsy from 26 subjects with a clinical and neuropathological diagnosis of PD ($n=9$) and AD ($n=8$), as well as age-matched controls ($n=9$). There were no differences in age at the time of death ($p=0.28$) or postmortem interval ($p=0.47$) among the three groups examined (Table 1). All patients with PD were diagnosed by neurologists in the Section of Movement Disorders in the Department of Neurological Sciences at Rush University Medical Center. Post mortem, the clinical diagnosis was confirmed by neuropathologists at Rush University Medical Center. For PD, inclusion criteria included a history compatible with idiopathic PD and at least two of the four cardinal signs (rest tremor, rigidity, akinesia/bradykinesia, and gait disturbance/postural reflex impairment). The Unified Parkinson's Disease Rating Scale3 (UPDRS3 "on") and Hoehn and Yahr staging (H&Y "on") were recorded. The pathological diagnosis was based on finding Lewy bodies in catecholamine nuclei such as the SN. Exclusion criteria included familial PD, dementia with Lewy bodies, the Lewy body variant of AD, or the combination of PD and AD. AD and age-matched control subjects were all participants in the Religious Order Study, a longitudinal clinical–pathological study of aging and AD, comprised of older Catholic nuns, priests, and brothers. Each participant received a clinical evaluation that included an assessment for movement disorders. Details of the clinical evaluation were reported previously (Chu et al., 2006). A team of investigators led by a neurologist performed a complete clinical evaluation annually. The pathological diagnosis in these cases was made by a neuropathologist at Rush University Medical Center. Subjects without neurological or psychiatric illnesses were included in the control group. AD was diagnosed clinically by the examining neurologist and by demonstration of impairment on neuropsychological testing. The study was approved by the Human Investigation Committee at Rush University Medical Center. All cases were evaluated for AD pathology according to National Institute on Aging/Reagan Institute Criteria.

At autopsy, the brains were removed from the calvarium and processed as described previously (Chu et al., 2006, 2009). Briefly, each brain was cut into 1 cm coronal slabs using a Plexiglas brain slice apparatus and then hemisected. Tissue punched from the SN was frozen and stored at −80 °C for western blot analysis. The slabs were fixed in 4% paraformaldehyde for 48 h at 4 °C. The left side brain slabs were used for pathological diagnoses. The right side brain slabs were cryoprotected in 0.1 M phosphate buffered saline (PBS; pH 7.4) containing 2% dimethyl sulfoxide (DMSO), 10% glycerol for 48 h followed by 2% DMSO, 20% glycerol in PBS for at least 2 days prior to sectioning. The fixed slabs containing the SN were cut into 18 adjacent series of 40 μm thick sections on a freezing sliding microtome for this study. All sections were collected and stored in a cryoprotectant solution prior to processing.

Table 1
Summary of case demographics.

	Age (years)	Gender	PMI (h)	UPDRS III	H&Y
Parkinson's disease case no.					
1	76	F	5.0	36	2
2	77	F	6.0	63	5
3	71	F	11.5	69	5
4	63	F	5.0	58	5
5	81	M	3.1	39	4
6	78	M	4.5	72	4
7	63	M	3.0	51	5
8	95	M	11.0	62	5
9	88	M	N/A	7	2
Mean ± SE	76.89 ± 3.51		6.14 ± 1.17	50.78 ± 6.86	4.10 ± 0.42

	Age (years)	Gender	PMI (h)	MMSE
Alzheimer's disease case no				
1	80	M	3.0	15
2	84	F	12.0	21
3	75	F	4.0	27
4	84	F	4.0	25
5	82	F	5.3	21
6	82	F	2.1	16
7	92	F	3.0	4
8	85	F	2.0	25
Mean ± SE	83.00 ± 1.70		4.43 ± 1.15	19.25 ± 2.65***
Age-matched control no				
1	88	F	8.0	28
2	71	M	4.1	29
3	73	M	7.3	28
4	86	F	3.8	27
5	85	M	4.0	26
6	74	M	10.3	27
7	84	M	4.0	28
8	83	M	5.5	26
9	79	F	5.8	29
Mean ± SE	80.33 ± 62.09		5.87 ± 0.75	27.56 ± 0.38

PMI, postmortem interval; MMSE, mini-mental status examination; UPDRS, United Parkinson's Disease Rating Scale; H&Y, Hoehn and Yahr; ***$P<0.01$ compared with age-matched control.

Animals and surgery

Young adult Sprague–Dawley rats (both male and female; Charles River Laboratories, Wilmington, MA) were housed two to a cage with *ad libitum* access to food and water during a 12 h light/dark cycle, all animal experiments were approved by the Rush University Institutional Animal Care and Use Committee. Recombinant adeno-associated virus serotype 6 vector encoding human mutant (A30P) α-synuclein gene (rAAV-h-A30P) and green fluorescent protein gene (rAAV-GFP) were prepared and titered as described previously (Towne et al., 2008). Under xylazine/ketamine anesthesia, 2 μl of the vector suspension was injected stereotaxically into the right nigral region (5.3 mm posterior and 2.3 mm lateral to bregma; 7.7 mm ventral to dura). The needle was kept in place for an additional 5 min before slowly being withdrawn. At 6 weeks after injection, animals (rAAV-h-A30P, $n=10$; rAAV-GFP, $n=8$) were perfused through the ascending aorta with physiological saline, followed by 4% ice-cold paraformaldehyde. The brains were postfixed in the same solution for