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Research Paper

Bimolecular Fluorescence Complementation of Alpha-synuclein Demonstrates its Oligomerization with Dopaminergic Phenotype in Mice

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ABSTRACT

Alpha-synuclein (αSyn) is encoded by the first causal gene identified in Parkinson's disease (PD) and is the main component of Lewy bodies, a pathological hallmark of PD. aSyn-based animal models have contributed to our understanding of PD pathophysiology and to the development of therapeutics. Overexpression of human wildtype αSyn by viral vectors in rodents recapitulates the loss of dopaminergic neurons from the substantia nigra, another defining pathological feature of the disease. The development of a rat model exhibiting bimolecular fluorescence complementation (BiFC) of αSyn by recombinant adeno-associated virus facilitates detection of the toxic αSyn oligomers species. We report here neurochemical, neuropathological and behavioral characterization of BiFC of αSyn in mice. Overexpression and oligomerization of αSyn through BiFC is detected by conjugated fluorescence. Reduced striatal dopamine and loss of nigral dopaminergic neurons are accompanied neuroinflammation and abnormal motor activities. Our mouse model may provide a valuable tool to study the role of αSyn in PD and to explore therapeutic approaches.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder manifested by slowness in movement, muscular rigidity, rest tremor, postural and gait impairment, and non-motor features. Available dopaminergic treatments offer symptomatic relief. However, there is currently no therapy to slow, halt, or reverse its progressive course. Pathologically, loss of dopaminergic neurons in the substantia nigra (SN) and presence of Lewy bodies and Lewy neurites in the residual neurons are hallmarks of PD (Braak and Del Tredici, 2009). The main component of Lewy bodies is insoluble aggregates of alpha-synuclein (αSyn), a ubiquitously expressed neuronal protein. While native state and functions of αSyn are not completely understood, increasing evidence suggests that soluble oligomeric αSyn is the most toxic species (Dehay et al., 2015; Kingwell, 2017). Although the etiology of PD is unclear, neuroinflammation appears to be an important contributor to its pathogenesis (Hirsch et al., 2005; Hirsch and Hunot, 2009; Hirsch and Vyas, 2012).

αSyn is encoded by *SNCA*, the first gene identified to cause PD (Spillantini et al., 1997). Multiple copies and point mutations of *SNCA* lead to the early onset of familial PD, and αSyn also contributes the basis of genetic risk of developing sporadic PD (Kalinderi et al., 2016). Given the central role of αSyn in PD genetics and pathogenesis, various animal models overexpressing either wildtype (WT) or mutant forms of αSyn have been developed to model the disease and to develop therapies. Among these models, viral vector-mediated overexpression of αSyn offers several advantages in recapitulating dopaminergic pathology of PD (Koprich et al., 2017). Using targeted overexpression of human WT αSyn in the SN by recombinant adeno-associated virus (AAV), we and others demonstrated loss of nigral dopaminergic neurons and neuroinflammatory responses in both rats and mice (Harms et al., 2013; McFarland et al., 2009; Theodore et al., 2008). The development of a bimolecular fluorescence complementation (BiFC) assay in rats facilitates direct detection of overexpression of αSyn fused to the N- and C- terminus half of venusYFP and formation of αSyn oligomers. The rat model shows striatal gliosis, neuritic dystrophy and loss of nigral dopaminergic neurons (Dimant et al., 2013).

Here we report systematical characterization of this BiFC system in mice including neuropathological, neurochemical, synucleinopathic, neuroinflammatory and behavioral changes. Time courses and dose responses were explored, with comparison to the original non-BiFC system. Since mice are the most commonly used animal species in PD research and most genetic probes are readily available in mice, our mouse model may provide a valuable tool to explore new therapeutic approaches for PD.

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2. Materials and Methods

2.1. Mice

Adult C57BL/6J mice (4–5 months old, male and female, weighing 25–30 g) from the Jackson laboratory were used for all experiments. Animals were randomly assigned to different groups. Animals were maintained in home cages at constant temperature with a 12-h light/dark cycle and free access to food and water. All experiments were performed in accordance with a protocol approved by the Massachusetts General Hospital Animal Care and Use Committee and in compliance with the National Institute of Health guidelines for the use of experimental animals.

2.2. Viral Vectors

BiFC vectors: (1) pAAV-CBA-Venus1-human αSyn -WPRE (V1S) by inserting the human WT *SNCA* fused with N-terminus half of venusYFP into the *Eco*RV and *Nhe*I sites of pAAV-CBA-WPRE vector; (2) pAAV-CBA- human αSyn -venus2-WPRE (SV2) by inserting the human WT *SNCA* fused with C-terminus half of venusYFP into the EcoRV and NheI sites of pAAV-CBA-WPRE vector; (3) pAAV-CBA-venusYFP-WPRE (venus) by inserting the venusYFP into the *Xho*I and NheI sites of pAAV-CBA-WPRE vector (Dimant et al., 2013).

Non-BiFC vectors: (1) pAAV-CBA- human αSyn -WPRE (αSyn) by inserting the human WT *SNCA* into the XhoI and NheI sites of pAAV-CBA-WPRE vector (St Martin et al., 2007; Theodore et al., 2008); (2) pAAV-CBA-WPRE empty vector (vector).

All vectors were packaged and purified in AAV serotype 8 by the Mayo Clinic Viral Vector laboratory.

2.3. Stereotaxic Virus Injections

Mice were anesthetized by intraperitoneal injection of Avertin and were placed in a stereotaxic frame. A total volume of 2 μl of virus was infused unilaterally at a rate of 0.1 ul/min into the left SN at coordinates at AP 0.09, ML 0.12, and DV -0.43 cm with lambda as a point of reference. At the end of the injection, the needle remained in place for 5 min before gradual removal.

The final injection titers (genome copies (gc)/ml) were: 5.1×10^{12} for V1SSV2 (mixing V1S at 4.7×10^{12} and SV2 at 5.4×10^{12} with equal volumes); 0.6×10^{12} and 4.4×10^{12} for venus. 3.9×10^{12} and 7.8×10^{12} for αSyn; 7.7×10^{12} for vector.

Authors who performed viral injections were blind to vector group information. Sample sizes were determined by power calculation to provide 80% power to detect 20–30% biologically meaningful changes in primary outcome measure (nigral dopaminergic neuron counts) based on our published estimates of mean ± SEM among WT (Chen et al., 2013) and one-way ANOVA with Tukey post-hoc test at $p < 0.05$.

2.4. Behavioral Testing

Locomotor activity was assessed using the open field test as described (Chen et al., 2017; Graham and Sidhu, 2010). Mice were placed in the activity chamber (11 × 11 in. with clear 8-in. high walls) and monitored by an infrared video tracking system for 10 min (Ethovision XT 9.0, Noldus Information Technology, The Netherlands). Tests were conducted in the first hour of the dark cycle on two consecutive days. Averages of the distance traveled, active time duration and velocity from the two sessions were calculated.

Amphetamine-induced (5 mg/kg *i.p.*) rotational behavior was assessed in an automated rotometry system (San Diego Instruments) for 60 min as described previously (Chen et al., 2017).

2.5. Immunostaining and Quantitative Analysis

Animals were sacrificed, and their brains were dissected, post-fixed in 4% paraformaldehyde overnight, cryoprotected in 30% sucrose and sectioned coronally as previously described (Chen et al., 2017). Sections were processed accordingly and incubated with primary antibodies. Primary antibodies were: mouse monoclonal antibody against αSyn (Thermo Fisher Scientific Cat# AHB0261, RRID:AB_2536241, at 1:500), mouse monoclonal antibody against pSer129-αSyn (BioLegend Cat# 825701, RRID:AB_2564891, at 1:500), mouse monoclonal antibody against astrocytes marker glial fibrillary acidic protein (GFAP, Sigma-Aldrich Cat# G3893, RRID:AB_477010, at 1:2500), rabbit monoclonal antibody against microglia marker ionized calcium-binding adapter molecule 1 (iba-1, Abcam Cat# ab178846, RRID:AB_2636859, at 1:2000), and rabbit polyclonal antibody against dopaminergic neuron marker tyrosine hydroxylase (TH, Enzo Life Sciences Cat# BML-SA497-0100, RRID:AB_2052772, at 1:1000). For peroxidase staining, sections were incubated with appropriate secondary antibodies and the staining was developed by incubating with 3,3′-Diaminobenzidine (DAB). For fluorescent TH staining, sections were incubated with goat anti-rabbit lgG-Alexafluor 546 Alexa (Thermo Fisher Scientific Cat# A-11010, RRID:AB_2534077, at 1:1000). Sections were covered by ProLong™ Gold Antifade Mountant with 4′,6-diamidino-2-phenylindole (DAPI) nuclear staining after serial washes.

For analysis of GFAP staining and YFP fluorescence intensity, images were captured under an Olympus BX50 microscope (Olympus Optical Co., Tokyo, Japan) with a DP 70 digital camera system using the same camera gain, exposure time and pixel setting for all sections. Integrated optical density (IOD) of GFAP staining in images taken under ×40 objective was analyzed by Image J. Two midbrain sections containing the central and anterior SN per mouse were analyzed. YFP fluorescence average intensity above background in the ipsilateral SN was analyzed by ImageJ using images taken under ×10 objective.

Morphology of iba1-positive cells in the SN pars compacta (SNpc) was analyzed and classified according to the published method by Sanchez-Guajardo et al. (2010). Based on their morphological characteristics, iba1-positive cells were classified as resting (type A, visible thin cytoplasm with long and thin processes), activated (type B, dense and enlarged cell body with thick, short processes), and phagocytic (type C, pseudo-amoeboid shape, big, dark cell body merging with processes) microglia. Cells were classified and counted cell by cell using stereological method at 40× magnification (Olympus BX51 microscope and Olympus CAST stereology software) as previously described (Dimant et al., 2013; West et al., 1991). Two midbrain sections containing the central and anterior SN per mouse were analyzed. Percentage of each cell type was calculated.

2.6. Stereological Analysis of Nigral Dopaminergic Neurons

For dopaminergic neuron counts, a complete set of serial SN sections at 30 μm from each animal was stained for TH and counterstained for Nissl substance (Chen et al., 2013, 2017). Sections were coded and the number of TH-positive cells was counted by unbiased stereology based on the optical fractionator principle using Olympus BX51 microscope and Olympus CAST stereology software, as previously described (Dimant et al., 2013; West et al., 1991).

2.7. Proteinase K Digestion

Proteinase K (PK) digestion was performed as previously reported (Dimant et al., 2013). Briefly, brain sections were mounted on the slides, and dry sections were rehydrated in TBS buffer containing 0.05% tween-20 (PH7.4). Sections were then treated with 50 μg/ml PK at 55 °C for 120 min.

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