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Apoptosis signal regulating kinase 1 deletion mitigates α-synuclein pre-formed fibril propagation in mice

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ABSTRACT

α-Synuclein (α-Syn) is a key pathogenic protein in α-synucleinopathies including Parkinson disease and dementia with Lewy bodies. Accumulating evidence has shown that misfolded fibrillar α-Syn is transmitted from cell-to-cell, a phenomenon that correlates with clinical progression of the disease. We previously showed that deleting the MAP3 kinase apoptosis signal-regulating kinase 1 (ASK1), which is a central player linking oxidative stress with neuroinflammation, mitigates the phenotype of α-Syn transgenic mice. However, whether ASK1 impacts pathology and disease progression induced by recombinant α-Syn pre-formed fibrils (PFF) remains unknown. Here, we compared the neuropathological and behavioral phenotype of ASK1 knock-out mice with that of wild-type mice following intrastriatal injections of α-Syn PFF. At 6 months post-injections, ASK1 null mice exhibited reduced amount of phosphorylated α-Syn aggregates in the striatum and cortex, and less pronounced degeneration of the nigrostriatal pathway. Additionally, the neuroinflammatory reaction to α-Syn PFF injection and propagation seen in wild-type mice was attenuated in ASK1 knock-out animals. These neuropathological markers were associated with better behavioral performance. These data suggest that ASK1 plays an important role in pathological α-Syn fibril transmission and, consequently, may impact disease progression. These findings collectively support inhibiting ASK1 as a disease modifying therapeutic strategy for Parkinson disease and related α-synucleinopathies.

1. Introduction

Parkinson disease (PD) is a chronic, progressive neurological disorder diagnosed based on characteristic motor symptoms that develop as a result of loss of dopaminergic neurons in the substantia nigra pars compacta (SNc). The neuropathological picture includes intraneuronal aggregates of fibrillar α-synuclein (α-Syn) in hallmark Lewy bodies and Lewy neurites (Dickson et al., 2009; Spillantini et al., 1998). The oligomerization and aggregation of the normally intrinsically disordered protein α-Syn lead to neuronal dysfunction and death (Goedert, 2001; Ma et al., 2003). Aggregates of α-Syn can spread to anatomically connected brain regions (Luk et al., 2012), a phenomenon that correlates with the clinical progression of the disease and the emergence of additional neuropsychiatric manifestations as the disease advances (Braak and Del Tredici, 2017). These pathologic aggregates are typically hyperphosphorylated at serine 129, and antibodies raised against phospho-α-Syn (p-α-Syn) are commonly used to detect inclusions (Anderson et al., 2006; Fujiwara et al., 2002; Oueslati, 2016).

Accumulating evidence suggests that oxidative stress plays a role in the neurodegeneration of PD (Dias et al., 2013). Several PD-linked gene products, including α-Syn, are associated with and contribute to mitochondrial dysfunction leading to reactive oxygen species (ROS) generation (Dias et al., 2013; Hwang, 2013; Junn and Mouradian, 2002; Langston et al., 1999; Sedelis et al., 2000). Chronic inflammation is also a feature of the PD pathology.

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Inflammatory responses manifested by glial reaction, T cell infiltration, and increased expression of inflammatory cytokines, as well as other toxic mediators derived from activated microglia, are recognized features of PD affected brains (Beraud and Maguire-Zeiss, 2012; Blandini, 2010; Cornejo Castro et al., 2010; Glass et al., 2010; Halliday and Stevens, 2011; Saijo and Glass, 2011; Valente et al., 2012). Notably, the confluence of oxidative stress (Grassi et al., 2018), neuroinflammation (Blumenstock et al., 2017; Boza-Serrano et al., 2014; Sacino et al., 2014), and phosphorylated α-Syn aggregates are reproduced in the exogenous, recombinant α-Syn pre-formed fibrils (PFF) inoculation model in the striatum, in which endogenous α-Syn is nucleated and propagates to synaptically connected brain regions including nigral dopaminergic neurons (Luk et al., 2012; Yan et al., 2018).

Apoptosis signal-regulating kinase 1 (ASK1) is a member of the MAP3 kinase family that is activated in response to various stimuli including oxidative stress (Saitoh et al., 1998; Song et al., 2002), leading to apoptosis through JNK and p38 kinase pathways (Ichijo et al., 1997; Tobiume et al., 2001). Consistent with the state of oxidative stress in PD, ASK1 is found to be activated in SNc dopaminergic neurons of PD affected brains (Hu et al., 2011). Experimentally, challenging mice with α-Syn overexpression in transgenic mice (Lee et al., 2015) or with the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Lee et al., 2012) results in activation/phosphorylation of ASK1 in the SNc. On the other hand, ASK1 null mice exhibit attenuated dopaminergic neuronal loss, inflammatory responses, and motor impairment induced by α-Syn overexpression or MPTP (Lee et al., 2012, 2015). ASK1 null primary neurons are also resistant to cell death brought about by endoplasmic reticulum stress and proteasome dysfunction (Nishitoh et al., 2002). And knocking down ASK1 in the SNc of mice using a lentiviral vector expressing short hairpin interfering RNA against ASK1 protects against 6-hydroxydopamine toxicity (Hu et al., 2011). Thus, ASK1 signaling plays an important role in the pathogenesis of PD by modulating responses to both α-Syn and oxidative stress.

Considering that α-Syn PFF inoculation induces oxidative stress and neuroinflammation, while ASK1 deletion mitigates these 2 important pathogenetic phenomena, the present study addresses the role of ASK1 in the nucleation and propagation of α-Syn fibrils in the mouse brain. Our findings show that ASK1 deletion attenuates the accumulation and propagation of hyperphosphorylated α-Syn aggregates, reduces the inflammation, and protects nigrostriatal dopaminergic neurons following α-Syn PFF inoculation. All these lead to improved behavioral performance. These findings collectively raise the possibility that ASK1 provides a target for developing therapeutics designed to slow the progression of PD and related α-synucleinopathies.

2. Materials and methods

2.1. Animals

C57BL/6J mice were obtained from the Jackson Laboratories (Bar Harbor, ME, USA). ASK1 knockout mice ($ASK1^{KO}$) are described previously (Lee et al., 2012; Tobiume et al., 2001). Male mice were used for these studies because of practicability, as female mice were used for breeding and more male mice were available that could be used for 8-month long studies. All housing, breeding, and procedures were performed according to the NIH Guide for the Care and Use of Experimental Animals and approved by the Rutgers Robert Wood Johnson Medical School Institutional Animal Care and Use Committee.

2.2. Preparation of recombinant α-Syn fibrils

Recombinant α-Syn fibrils were prepared as described previously (Yan et al., 2018). pT7-7 plasmid-expressing mouse α-Syn cDNA (Weinreb et al., 1996; Wu et al., 2008) was used to transform *Escherichia coli* BL21 (DE3) strain (Invitrogen Inc), and the bacteria were cultured in 1 L of lysogeny broth at 37 °C. One milliliter of 1 M isopropyl β-D-1-thiogalactopyranoside was added to induce α-Syn expression when the OD600 reading reached 0.8. After 4 hours of continued culture, cells were collected by centrifugation at 2000 *g* for 30 minutes, and the pellet was resuspended in 25 mL phosphate buffered saline (PBS) and homogenized 3 times by EmulsiFlex-C5 Homogenizer (AVESTIN). Lysate was then centrifuged at 24,000 *g* for 30 minutes. Supernatant was collected and 10 mg/mL streptomycin sulfate was added. Mixed solution was stirred at 4 °C for 30 minutes and then centrifuged at 24,000 *g* for 30 minutes. Supernatant was collected, and 0.361 g/mL ammonium sulfate was added. Then the solution was stirred at 4 °C for 1 hour and centrifuged at 24,000 *g* for 30 minutes. The pellet was collected and resuspended in 15 mL PBS and then boiled in a water bath for 15 minutes. After cooling, the sample was centrifuged at 24,000 *g* for 30 minutes and the supernatant was collected and dialyzed into 25 mM Tris buffer (pH 7.7). α-Syn was then separated by fast protein liquid chromatography (GE Healthcare) using 5 mL anion exchange column HiTrap Q (GE Healthcare) and eluted with NaCl (30%, 50%, and 100% gradient). Solution containing α-Syn was then dialyzed against ammonium bicarbonate before lyophilization and the freeze-dried α-Syn was dissolved in PBS at 5 mg/mL. Purified α-Syn was then allowed to shake at 1000 rpm at 37 °C for 7 days in a ThermoMixer C (Eppendorf). Formation of α-Syn fibrils was monitored and confirmed using thioflavin T aggregation assay (Fig. S1A) (Lee et al., 2015). Fibrils were then aliquoted and stored at −80 °C, and used within 4 months of preparation. The stability of α-Syn fibrils after storage for 4 years was confirmed by transmission electron microscopy, showing no ultrastructural difference and no obvious oligomers (Fig. S1C and D).

2.3. Stereotaxic injections

α-Syn PFF preparation was diluted in sterile PBS and sonicated at 60% power 30 times with a hand-held probe (Sonics & Materials Inc, Danbury, CT) before intracerebral injection of 2-month-old mice. C57BL/6J wild-type (WT) or $ASK1^{KO}$ mice were anesthetized with ketamine hydrochloride (100 mg/kg, intraperitoneally (i.p.)) and xylazine (10 mg/kg, i.p.). Ten microgram of recombinant α-Syn PFF was stereotaxically injected into the right striatum at 2 sites (anterior–posterior: 0.2 mm; medial–lateral: −2.0 mm; dorsal–ventral: −2.6 mm; and anterior–posterior: 0.8 mm; medial–lateral: −1.5 mm; dorsal–ventral: −3.5 mm) (Luk et al., 2012). Control animals received sterile PBS. Injections were performed at a rate of 0.2 μL/min (total 2.5 μL per site) using a 10 μL Hamilton neuros syringe equipped with a 33 gauge needle and attached to a Quintessential Stereotaxic Injector (Stoelting, Wood Dale, IL, USA). To prevent reflux, after each infusion, the injection needle was left in place for 5 minutes before removal. Animals were sacrificed 6 months post injections.

2.4. Immunohistochemistry and immunofluorescence

Mice were perfused transcardially with PBS, and the brains were removed and fixed in 10% formalin (Sigma) at 4 °C overnight. Brains were sectioned using a cryostat at 20 μm thickness, and slices were collected as sets with the same interval. For

immunohistochemistry, free-floating sections were blocked by 1% bovine serum albumin and 0.2% Triton X100 in PBS. Sections were then incubated with primary antibody at 4 °C overnight and biotinylated secondary antibody for 1 hour at room temperature. Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) and 3.3′-diaminobenzidine (Sigma-Aldrich) were used for amplifying and color development. Images were captured by a Nikon Eclipse 55i microscope. Staining intensity and phosphorylated α-synuclein (p-α-Syn) aggregate counts were obtained by Image-Pro Plus. Intensity calibration was set to the level of a blank area in each image. Hue, saturation, and intensity (HSI) was used for color selection with the standard parameters of H: 0–30, S: 0–255, and I: 0–160. To count only positively stained cells, color selection was adjusted according to the antibody used and background intensity. Stains that were smaller than 4 pixels were excluded from analysis. For striatal tyrosine hydroxylase (TH) staining, an elliptical area of interest of the same size that encompasses the striatum was applied to all the sections. In the SNc, 4 images covering the whole SNc per brain were counted. For p-α-Syn stains in the cortex, 4 matching regions in each of 2 sections per animal were counted. In the dorsal striatum, 3 matching regions in each of 3 sections per brain were counted. For Iba-1 staining in the striatum, 4 matching regions in each of 4 sections per brain were analyzed. For immunofluorescence staining of dopamine (DA), 20-μm thick cryostat sections were blocked with 5% goat serum and 0.2% Triton X-100 in PBS. Sections were then incubated with primary antibody overnight at 4 °C and fluorescent secondary antibody for 1 hour at room temperature. Images were captured using Carl Zeiss Axiovert 200 microscope. For DA intensity analysis, a rectangular area of interest of the same size that encompasses the striatum was applied to all the sections. Primary antibodies used were anti-phospho-Ser129-α-Syn (#015-25191; WAKO), anti-Iba-1 (#019-19741; WAKO), anti-TH (T2928; Sigma), rabbit phospho-ASK1 generated against the ASK1 epitope that contains p-Thr845 (Lee et al., 2012; Tobiume et al., 2002), and anti-DA (IS1005; ImmuSmol).

2.5. Cell culture and Western blot analysis

Human neuroblastoma SH-SY5Y cells were cultured in Dulbecco's Modified Eagle Medium/F-12 with 10% FBS. Cells were plated in 12-well tissue culture plates and cultured for 24 hours to reach 80% confluency (the first day). Transfection of plasmid or small interferring RNA was done on the second day. For ASK1 overexpression experiment, cells were treated with 2 and 5 μg/mL mouse α-Syn PFF in Dulbecco's Modified Eagle Medium/F-12 on the third day and harvested on the fifth day (α-Syn PFF challenge for 2 days). For ASK1 knockdown experiment, small interferring RNA was added 2 additional times on days 4 and 6, and cells were harvested on the eighth day (α-Syn PFF challenge for 5 days). Medium was refreshed 24 hours after each transfection. Cells were lysed in a buffer containing PBS with 1% Triton X-100 and a mixture of protease inhibitor (Millipore). After incubation on ice for 30 minutes, cells were centrifuged at 20,000 *g* at 4 °C for 30 minutes. Triton X-100 insoluble pellets were dissolved in PBS containing 2% sodium dodecyl sulfate and mixture of protease inhibitors with sonication. Insoluble fractions were used in Western blot analysis. Protein concentration was determined by bicinchoninic acid method (Pierce), equal amount of protein was separated on NuPage 4%–20% SDS-polyacrylamide gel electrophoresis gel (GenScript), and transferred to polyvinylidene fluoride membrane (Bio-Rad). Membranes were blocked with 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) in Tris-buffered saline and 0.1% Tween-20 before probing with antibodies. ECL Plus (Perkin-Elmer, Waltham, MA, USA) was used to develop immunoblots. Primary antibodies used were anti-α-synuclein (#610787; BD Bioscience), ASK1 (Cell Signaling Technology, Danvers, MA, USA), myc-horseradish peroxidase (Santa Cruz Biotechnology, Dallas, TX, USA), and β-actin (Sigma-Aldrich).

2.6. Behavioral assessments

Behavioral assessments were performed at 3 months and 6 months post α-Syn PFF injections. To test nesting behavior, each mouse was housed separately in a cage with a 5-cm square cotton nestlet (Ancare, Bellmore, NY, USA). Twenty-four hours later, the mouse was removed, a photograph of the cage was taken, and nest formation was rated by a rater blinded to the genotype or injection group of the mice on a half-point scale from 1 (non-shredded) to 5 (maximally shredded) (Deacon, 2006). For the rotarod test, mice were placed on a rotating cylinder (diameter = 4.5 cm) with a coarse surface for firm grip and were trained for 4 trails for the first 4 days: the first 2 trials are acquisition trials with increasing speed from 4 to 20 rpm in 180 seconds, the last 2 trials are the actual probe trials with an accelerating speed of 0.2 rpm/s, increasing from 4 to 40 rpm in 180 seconds. On the fifth day, mice were tested for 3 actual probe trials, latency on the rod before falling was measured, and the average of 3 trials taken.

2.7. Statistical analysis

Data are presented as means ± standard error of the mean and analyzed by either 2-way analysis of variance followed by Tukey's multiple comparison test or unpaired *t*-test. Significance was determined at $p < 0.05$.

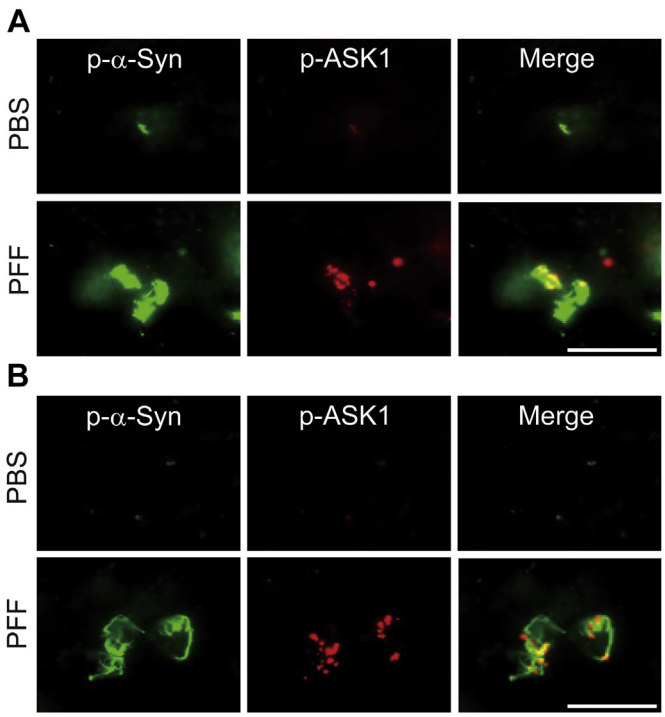

Fig. 1. Intrastriatal inoculation of synthetic murine α-Syn PFF activates ASK1 in WT mice. Activation/phosphorylation of ASK1 in the striatum (A) and cortex (B) of C57BL/6J mice after unilateral injection of α-Syn PFF into the dorsal striatum 6 months earlier. No phospho-ASK1 signal is detected in PBS injected brains. Data shown are representative of immunofluorescent stains of 5 brains. Scale bar = 50 μm. Abbreviations: α-Syn, α-synuclein; ASK1, apoptosis signal-regulating kinase 1; PPF, pre-formed fibril; WT, wild type.

3. Results

3.1. α-Syn PFF induces phosphorylation/activation of ASK1 in wild type mice

We previously showed that α-Syn overexpression leads to activation of ASK1 in both cultured PC12 cells and brains of α-Syn transgenic mice (Lee et al., 2015). To test whether ASK1 is also activated in the α-Syn PFF model (Luk et al., 2012; Paumier et al., 2015), we examined the state of ASK1 phosphorylation in the striatum (Fig. 1A) and cortex (Fig. 1B) of WT C57BL/6 mice inoculated intrastriatally with murine α-Syn PFF 6 months earlier. As expected, p-α-Syn and phospho-ASK1 were detected and co-localized in both brain regions in α-Syn PFF inoculated brains but not in PBS injected brains. This finding suggests that synthetic recombinant fibrils of α-Syn lead to ASK1 activation in vivo.

3.2. ASK1 deletion prevents the nucleation and propagation of p-α-synuclein positive aggregates

To examine whether ASK1 impacts p-α-Syn aggregation and propagation in the α-Syn PFF model, striatal and cortical tissue sections were stained with p-α-Syn antibody. As described previously (Luk et al., 2012), abundant aggregates were found in the ipsilateral striatum and cortex of α-Syn PFF inoculated WT mice, and fewer p-α-Syn immunoreactive neurons were detected in the contralateral hemisphere (Fig. 2A–D). On the other hand, ASK1 null mice injected with α-Syn PFF had less abundant p-α-Syn immunoreactivity not only in ipsilateral but also contralateral striatum (Fig. 2A, B, E, and F). Similarly, the number of p-α-Syn immunopositive neurons in the cortex of ASK1 null α-Syn PFF inoculated mice were fewer than that in WT α-Syn PFF inoculated mice (Fig. 2C, D, G, and H). PBS injected brains had no p-α-Syn

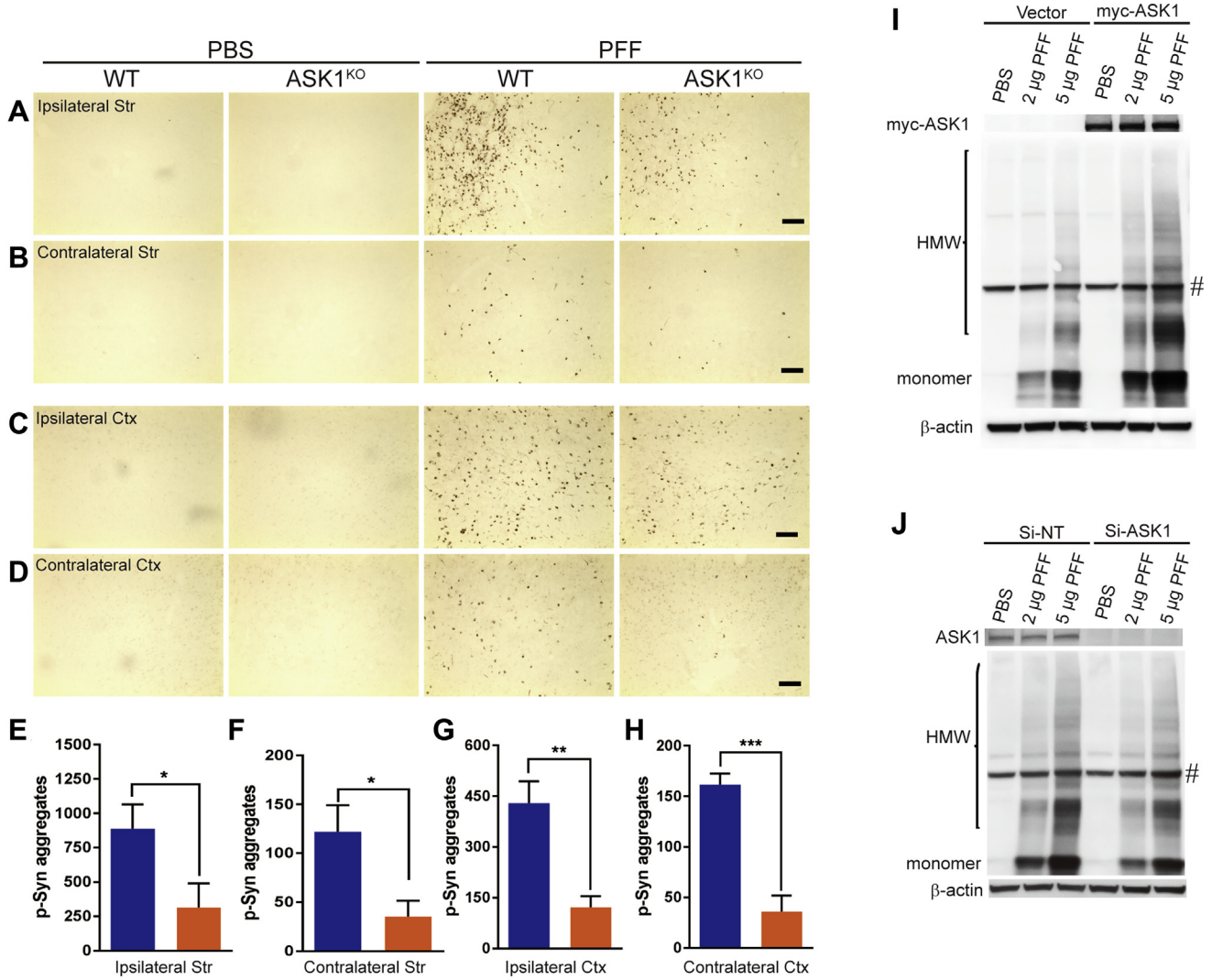

Fig. 2. ASK1 promotes the aggregation and propagation of phosphorylated α-Syn. Representative images of p-α-Syn staining in the ipsilateral and contralateral striatum (A, B), and ipsilateral and contralateral cortex (C, D) following unilateral intrastriatal injections of PBS or α-Syn PFF in WT or ASK1 null mice. (E–H) Quantification of p-α-Syn positive cells in WT (blue) and ASK1 null (orange) mice following intrastriatal α-Syn PFF injections. Data shown are means ± SEM ($n = 5$ mice per group). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, 2-tailed unpaired *t*-test. Scale bar = 100 μm. (I) SH-SY5Y cells engineered to express myc-ASK1 or transfected with an empty vector were challenged with the indicated concentrations of α-Syn PFF for 48 hours. myc-ASK1 expression was detected by anti-myc antibody. Overexpression of myc-ASK1 significantly increased high molecular weight (HMW) α-Syn. (J) SH-SY5Y cells transfected with ASK1 siRNA or nontargeting sequence (si-NT) were challenged with the indicated concentrations of α-Syn PFF for 6 days. Knocking down ASK1 reduced α-Syn HMW accumulation. Nonspecific band is indicated as # in panels I and J. Abbreviations: α-Syn, α-synuclein; ASK1, apoptosis signal-regulating kinase 1; Ctx, cortex; PBS, phosphate buffered saline; PPF, pre-formed fibril; SEM, standard error of the mean; siRNA, small interferring RNA; Str, striatum; WT, wild type. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

immunoreactive neurons in both WT and ASK1 null mice. These results suggest that deletion of ASK1 prevents the seeding and propagation of α-Syn PFF induced pathologic aggregates.

3.3. ASK1 promotes α-Syn aggregation in SH-SY5Y cell challenged with α-Syn PFF

To examine whether ASK1 impacts α-Syn aggregation in a cellular model, SH-SY5Y cells were engineered to overexpress or knockdown ASK1 followed by different concentrations (2 and 5 μg/mL) of α-Syn PFF challenge. As expected, insoluble high molecular weight (HMW) species of α-Syn was present in all samples treated with α-Syn PFF (Fig. 2I and J). Overexpression of ASK1 resulted in significant amount of HMW α-Syn compared to cells transfected with empty vector at both concentrations of α-Syn PFF (Fig. 2I). Conversely, a remarkable reduction in HMW α-Syn was noted in ASK1 knockdown cells compared with cells transfected with non-targeting sequence (si-NT) at both concentrations of α-Syn PFF (Fig. 2J). These results indicate that ASK1 impacts α-Syn aggregation in cells.

3.4. ASK1 deletion attenuates the neuroinflammation in the α-Syn PFF inoculation model

α-Syn PFF inoculation of brain tissue induces robust microglial activation (Blumenstock et al., 2017; Boza-Serrano et al., 2014), while ASK1 deletion mitigates the neuroinflammatory response in α-Syn transgenic mice and in the MPTP model of PD (Lee et al., 2012, 2015). To investigate the impact of ASK1 on neuroinflammation induced by α-Syn PFF inoculation, brain tissue sections were stained with the microglial marker Iba-1. Compared with the significant increase in the number of reactive microglia in the ipsilateral striatum (Fig. 3A and B) and cerebral cortex (Fig. 3C and D) of WT mice injected with α-Syn PFF, the microglial response to these fibril injections were repressed in ASK1 null mice. PBS injections in the striatum did not impact microglia numbers. No difference was found in the contralateral striatum or cerebral cortex (Fig. S2). These findings suggest that ASK1 deletion can mitigate the microglia-mediated neuroinflammatory response to α-Syn PFF.

3.5. ASK1 deletion protects nigral dopaminergic neurons in the α-Syn PFF inoculation model

To address whether deleting ASK1 protects nigral dopaminergic neurons, we first assessed the integrity of nigrostriatal terminals and striatal DA content following α-Syn PFF inoculation model. Immunohistochemical staining of the striatum for TH (Fig. 4A–C) revealed that α-Syn PFF injections reduced dopaminergic terminals in the ipsilateral striatum by 40% in WT mice compared to only 18% in ASK1 null mice, representing a 22% protection (Fig. 4A and B). No changes in TH immunoreactivity were detected in the contralateral striatum in either mouse groups. Similarly, immunofluorescence staining of DA showed a smaller decline of DA content in the striatum ipsilateral to α-Syn PFF inoculation in ASK1 null mice compared to WT α-Syn PFF inoculated mice (78% residual DA in ASK null mice compared to 58% in WT mice) (Fig. 4D and E). Finally, TH immunostaining of the midbrain showed marked degeneration of

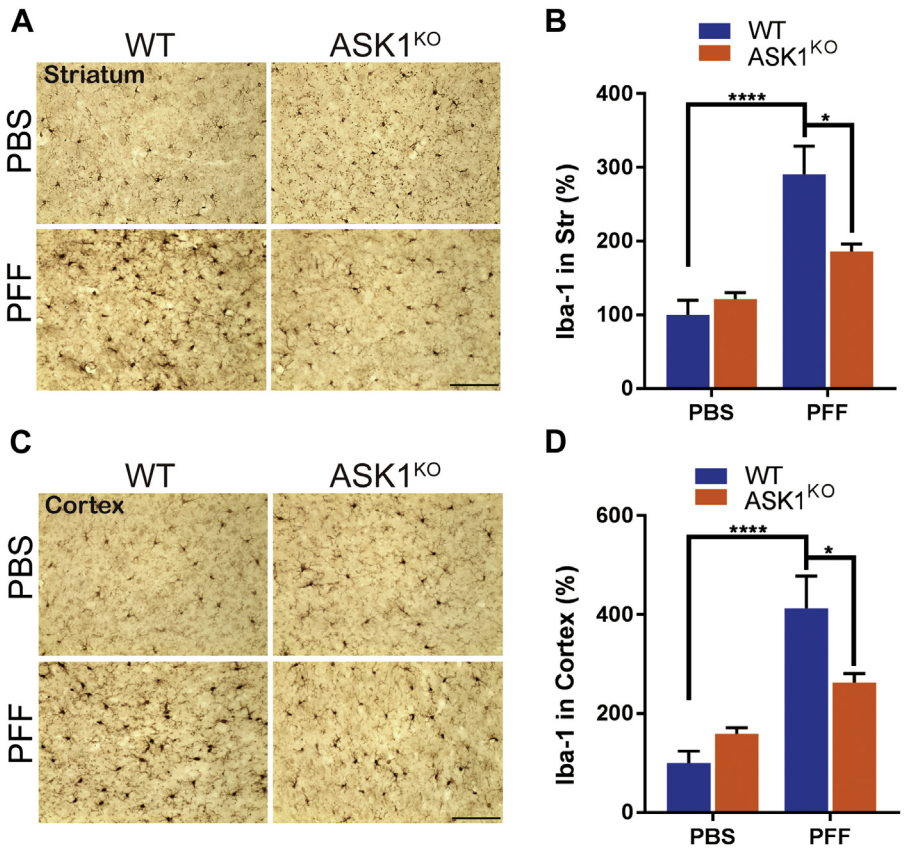

Fig. 3. ASK1 deletion suppresses the microglial activation that follows α-Syn PFF injections in WT mice. Representative immunohistochemistry images of the microglial marker Iba-1 in the ipsilateral striatum (A) and cortex (C) of WT or ASK1 null mice injected with α-Syn PFF or PBS in the right striatum. Quantification of microglial activation in the striatum (B) and cortex (D). Data shown are means ± SEM (n: WT + PBS = 6; WT + PFF = 5; $ASK1^{KO}$ + PBS = 6; $ASK1^{KO}$ + PFF = 5). *$p < 0.05$; ****$p < 0.0001$, 2-way analysis of variance. Scale bar = 100 μm. Abbreviations: α-Syn, α-synuclein; ASK1, apoptosis signal-regulating kinase 1; PBS, phosphate buffered saline; PPF, pre-formed fibril; SEM, standard error of the mean; WT, wild type.

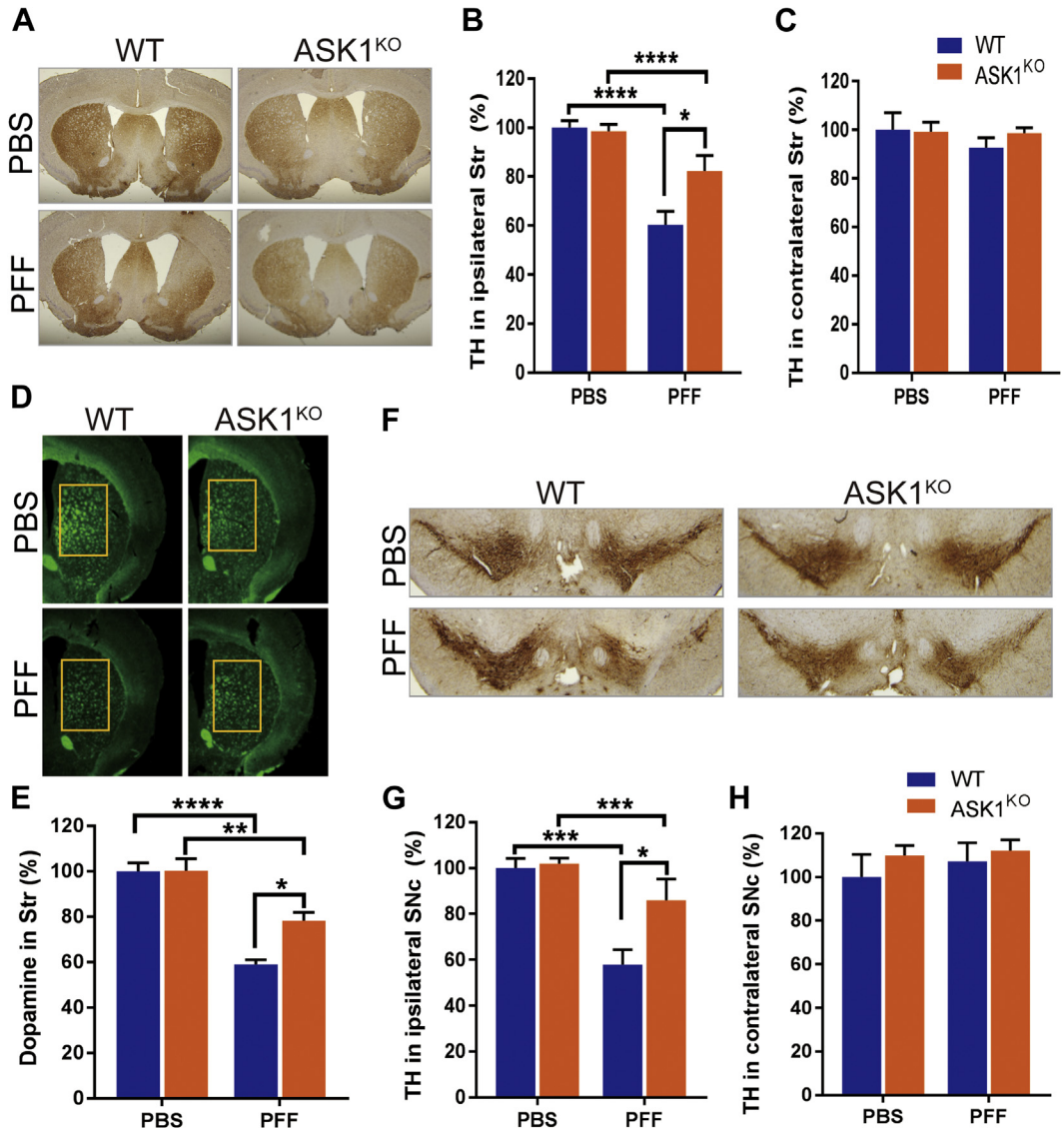

Fig. 4. ASK1 deletion protects nigral dopaminergic neurons against intrastriatal α-Syn PFF injections. (A) TH staining of the striatum (PBS or α-Syn PFF is injected in the right striatum) in WT and ASK1 null mice. Quantification of TH staining intensity in the ipsilateral (B) and contralateral (C) striatum of PBS versus α-Syn PFF-injected brains. (D) Dopamine staining of the striatum in PBS or α-Syn PFF injected hemisphere in WT or ASK1 null mice. (E) Quantification of dopamine staining intensity. (F) TH immunostaining of the substantia nigra (right is PBS or α-Syn PFF injected side) in WT and ASK1 null mice. Quantification of TH intensity in the substantia nigra of PBS versus α-Syn PFF injected ipsilateral (G) versus contralateral (H) hemisphere. Data shown are means ± SEM (n: WT + PBS = 6; WT + PFF = 5; $ASK1^{KO}$ + PBS = 6; $ASK1^{KO}$ + PFF = 5). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$, 2-way analysis of variance. Abbreviations: α-Syn, α-synuclein; ASK1, apoptosis signal-regulating kinase 1; PBS, phosphate buffered saline; PPF, pre-formed fibril; SEM, standard error of the mean; SNc, substantia nigra pars compacta; Str, striatum; WT, wild type.

ipsilateral nigral dopaminergic neurons in α-Syn PFF injected WT mice (43%) but a significantly attenuated decline in ASK1 null mice (14%) (Fig. 4F–H). These results collectively suggest that α-Syn PFF inoculation impairs dopaminergic neurons as well as the integrity of nigrostriatal pathway in WT mice, whereas deficiency of ASK prevents these consequences of α-Syn PFF.

3.6. Deleting ASK1 improves the behavioral performance of α-Syn PFF inoculated mice

To determine if the neuropathological markers of protection associated with ASK1 deletion correlate with improved behavior, nest building and performance on the rotarod test were assessed at 3 and 6 months post α-Syn PFF injections as described previously (Grosso et al., 2014; Lee et al., 2015). At 3 months, WT mice inoculated with α-Syn PFF showed significantly impaired nesting behavior and ability to stay on the rotarod compared to PBS injected mice, whereas the performance of ASK1 null mice inoculated with α-Syn PFF was unchanged (Fig. 5A and B). At 6 months, a similar decline in performance on both tests was seen in α-Syn PFF inoculated WT mice but not in ASK1 null (Fig. 5C and D). PBS injections had no effect on behavior in either group. These behavioral improvements in ASK1 null mice are consistent with the histopathologic data in Figs. 2–4.

4. Discussion

The present findings demonstrate that exogenously administered recombinant α-Syn fibrils lead to activation/phosphorylation of ASK1 in the brains of WT mice. Importantly, deleting ASK1 mitigates the phenotype of α-Syn PFF inoculation on a number of outcome measures including markedly attenuated accumulation

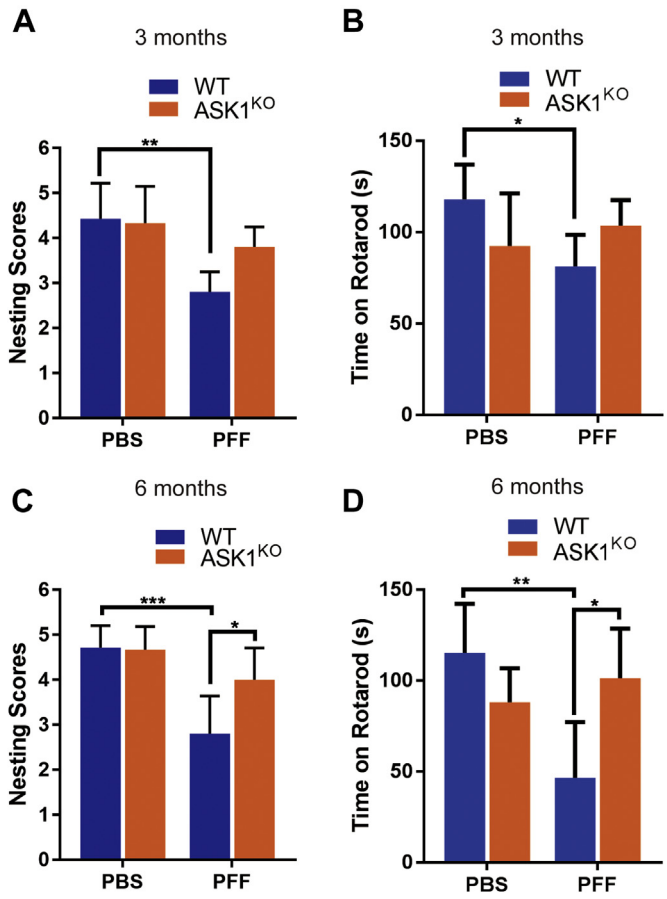

Fig. 5. Improvement in behavioral performance in ASK1 null mice compared with WT mice following α-Syn PFF injections. Performance on the nest building test at 3 months (A) and 6 months (C) post PBS versus α-Syn PFF injection. Performance on the rotarod at 3 months (B) and 6 months (D) post PBS versus α-Syn PFF injection. Data shown are means ± SEM (n: WT + PBS = 7; WT + PFF = 5; $ASK1^{KO}$ + PBS = 6; $ASK1^{KO}$ + PFF = 5). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, 2-way analysis of variance. Abbreviations: α-Syn, α-synuclein; ASK1, apoptosis signal-regulating kinase 1; PBS, phosphate buffered saline; PPF, pre-formed fibril; SEM, standard error of the mean; WT, wild type.

and propagation of hyperphosphorylated α-Syn aggregates, reduced neuroinflammation, and preserved integrity and function of SNc dopaminergic neurons. These are associated with better behavioral performance of α-Syn PFF inoculated ASK1 null mice compared to WT mice treated the same way with α-Syn PFF injections. These findings together suggest that ASK1 activation is an important step in the nucleation and propagation of α-Syn aggregates following exposure to exogenous fibrils.

ASK1 activation/phosphorylation by α-Syn PFF in the present study is consistent with our previous report showing that α-Syn overexpression leads to activation of ASK1 in both cultured PC12 cells and brains of α-Syn transgenic mice (Lee et al., 2015). Considering that α-Syn overexpression and α-Syn PFF induce ROS generation in vitro and in vivo (Dryanovski et al., 2013; Jiang et al., 2007; Junn and Mouradian, 2002; Turnbull et al., 2001), and oxidative stress is a potent activator of ASK1 (Saitoh et al., 1998; Song et al., 2002), we submit that elevated ROS levels induced by α-Syn PFF in the brain are the major factors that lead to ASK1 activation. This scenario is relevant to the PD affected brain where oxidative stress is well studied (Dias et al., 2013). In addition to pathologic α-Syn levels or aggregates leading to increased ROS generation in dopaminergic neurons, oxidant stress itself promotes α-Syn aggregate formation (Dryanovski et al., 2013; Goodwin et al., 2013). The absence of ASK1 may block this reciprocal cause and effect cycle and minimize the nucleation and propagation of pathologic protein aggregates. However, additional factors appear to underlie the impact of ASK1 activation on α-Syn aggregation, as reduced aggregation is evident in ASK1 null mice challenged with α-Syn PFF but not when cross-bred with α-Syn transgenic mice (Lee et al., 2015). It is conceivable that this difference relates to different paths toward aggregation between the 2 models: aggregation due to overexpression of monomeric α-Syn in transgenic mice compared to more complex processes involved in the PFF model including endocytosis, trafficking for lysosomal degradation, and contacts between exogenous seeds and endogenous α-Syn (Lee et al., 2014).

The role of α-Syn PFF in inducing microglia activation is well documented. α-Syn released from neurons activates the inflammatory response in a microglial cell line (Alvarez-Erviti et al., 2011), while microglia in turn exacerbate α-Syn-mediated neurotoxicity through phagocytosis of α-Syn and activation of nicotinamide adenine dinucleotide phosphate oxidase and ROS production (Zhang et al., 2005). Additionally, an inflammatory environment is known to enhance α-Syn aggregation, which can further activate microglia and promote a feed-forward cascade that leads to additional α-Syn aggregation and propagation (Gao et al., 2011). Several mechanisms have been suggested for mediating microglia activation by α-Syn fibrils (Boza-Serrano et al., 2014; Kim et al., 2009; Lee et al., 2010). In particular relevance to the present study, stereotactic injection of α-Syn protofibrils into the SNc of adult rats has been shown to result in microglia activation through p38 and the ERK1/2 MAP kinases pathway (Wilms et al., 2009). It is well known that ASK1 is upstream of p38 kinase activation, thus deleting ASK1 may block microglia activation through the p38 pathway. Our present finding that ASK1 deletion attenuates the microglia activation induced by α-Syn PFF injections suggests that ASK1 activation is required for this process in vivo.

The propagation of α-Syn aggregates across neurons is believed to underlie the progression of PD (Freundt et al., 2012; Masuda-Suzukake et al., 2014; Ulusoy et al., 2015). The mechanism by which α-Syn PFF leads to p-α-Syn nucleation and propagation remains to be fully understood. Evidence suggests that α-Syn PFF promotes the recruitment of endogenous soluble α-Syn into insoluble Lewy-like aggregates (Volpicelli-Daley et al., 2011). And this process can be accelerated by elevated levels of intraneuronal α-Syn. Adeno-associated virus-mediated overexpression of human α-Syn in the rat substantia nigra and ventral tegmental area 4 weeks prior to the delivery of α-Syn PFF in the same brain regions resulted in greater DA neuron loss, Lewy-like synucleinopathy, and neuroinflammation only 3 weeks post α-Syn PFF injections, compared to rats that were injected with α-Syn PFF alone (Thakur et al., 2017). These observations indicate that α-Syn PFF-induced nucleation and propagation is dependent on endogenous α-Syn in the brain, exacerbated by its overexpression, which itself induces greater oxidative stress and ASK1 activation (Dias et al., 2013; Saitoh et al., 1998; Song et al., 2002). Further investigations are needed to elucidate additional factors that might mediate the effect of ASK1 on nucleation and propagation.

In conclusion, our data provide evidence that ASK1 plays a role in mediating α-Syn-induced pathology, including α-Syn nucleation and propagation, microglia activation, and neuronal damage. Importantly, deleting ASK1 mitigates this pathological cascade. These findings extend and support our previous results that show ASK1 deletion diminishes the phenotype of α-Syn transgenic mice (Lee et al., 2015). Thus, the experimental evidence from 2 models of α-synucleinopathy collectively suggests that pharmacological inhibition of ASK1 may serve as a plausible strategy for the development of therapeutic interventions to slow or halt PD progression.

Recent progress in developing highly potent and selective ASK1 inhibitors (Lovering et al., 2018) can facilitate testing this hypothesis using appropriate brain penetrant compounds.

Disclosure

The authors have no actual or potential conflicts of interest.

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Author contributions: Zhang contributed to experimental design, carried out experiments, analyzed and interpreted data, and drafted the manuscript; E.S. Park contributed to experimental design, carried out experiments, and analyzed and interpreted data; H.J. Park carried out experiments, and analyzed and interpreted data; Yan, Zhang, and Yang carried out experiments and analyzed data; Grudniewska, Oh, and Baum analyzed and interpreted data; and Mouradian contributed to experimental design, analyzed and interpreted data, and wrote the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neurobiolaging.2019.09.012.

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