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Genetic diversity of axon degenerative mechanisms in models of Parkinson's disease

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ABSTRACT

Parkinson's disease (PD) is the most common form of neurodegenerative movement disorder, associated with profound loss of dopaminergic neurons from the basal ganglia. Though loss of dopaminergic neuron cell bodies from the substantia nigra *pars compacta* is a well-studied feature, atrophy and loss of their axons within the nigrostriatal tract is also emerging as an early event in disease progression. Genes that drive the *Wallerian* degeneration, like Sterile alpha and toll/interleukin-1 receptor motif containing (Sarm1), are excellent candidates for driving this axon degeneration, given similarities in the morphology of axon degeneration after axotomy and in PD. In the present study we assessed whether Sarm1 contributes to loss of dopaminergic projections in mouse models of PD. In Sarm1 deficient mice, we observed a significant delay in the degeneration of severed dopaminergic axons distal to a 6-OHDA lesion of the medial forebrain bundle (MFB) in the nigrostriatal tract, and an accompanying rescue of morphological, biochemical and behavioural phenotypes. However, we observed no difference compared to controls when striatal terminals were lesioned with 6-OHDA to induce a dying back form of neurodegeneration. Likewise, when PD phenotypes were induced using AAV-induced alpha-synuclein overexpression, we observed similar modest loss of dopaminergic terminals in Sarm1 knockouts and controls. Our data argues that axon degeneration after MFB lesion is Sarm1-dependent, but that other models for PD do not require Sarm1, or that Sarm1 acts with other redundant genetic pathways. This work adds to a growing body of evidence indicating Sarm1 contributes to some, but not all types of neurodegeneration, and supports the notion that while axon degeneration in many context appears morphologically similar, a diversity of axon degeneration programs exist.

Abbreviations: 6-OHDA, 6-Hydroxydopamine; AAV, Adeno-associated virus; ANOVA, Analysis of variance; AP, Anterior posterior; DA, Dopamine; dAMPH, *D-amphetamine*; *DOPAC*, *3,4-Dihydroxyphenylacetic acid*; DV, Dorsal ventral; Het, Heterozygous; HPLC, High performance liquid chromatography; HVA, Homovanillic acid; KO, Knock-out; LRRK2, Leucine-rich repeat kinase 2; MAPK, Mitogen-activated protein kinase; MFB, Medial forebrain bundle; ML, Medial lateral; NMNAT, Nicotinamide mononucleotide adenylyl-transferase; PBS, Phosphate buffered saline; PD, Parkinson's disease; PINK1, Phosphatase and tensin homolog (PTEN) induced putative kinase 1; PRKN, Parkin RBR E3 ubiquitin protein ligase; ROS, Reactive Oxygen Species; SARM1, Sterile alpha and toll/interleukin-1 receptor (TIR) motif containing 1; SEM, Standard error of mean; SNCA, Alpha synuclein; SNpc, Substantia nigra pars compacta; SV40, Simian virus 40; TH, Tyrosine hydroxylase; VTA, ventral tegmental area; Wld^S, Wallerian degeneration slow; WPRE, Woodchuck hepatitis virus post-transcriptional regulatory element; WT, Wild-type.

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1. Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, with symptoms primarily caused by profound loss of dopaminergic neurons from the basal ganglia. Though loss of dopaminergic neuron cell bodies from the substantia nigra *pars compacta (*SNpc*)* is well described, atrophy and loss of their axons within the nigrostriatal tract occurs early in disease progression (Kurowska et al., 2016). Suggestive of a retrograde mechanism of axon degeneration (Tagliaferro and Burke, 2016), neuritic aggregation of alpha-synuclein (Duda et al., 2002; Galvin et al., 1999), substantial reduction of dopaminergic terminals within the putamen (Kordower et al., 2013) and decreased levels of dopamine (Bernheimer et al., 1973; Cheng et al., 2010; Lee et al., 2000; Riederer and Wuketich, 1976) all occur early in disease, often preceding destruction of cell bodies. Dopaminergic neurons of the SNpc possess a vast, highly arborized axonal network that contacts many thousands of synaptic targets. The energetic and support demands of this critical neuronal compartment likely make the axons of DA neurons highly vulnerable to toxic insults underlying PD, with mitochondria dysfunction emerging as a likely contributing factor (Clark et al., 2006; Deng et al., 2008; Kitada et al., 1998; Valente et al., 2004).

Axon degeneration is emerging as a novel target for therapeutic intervention in the pathogenesis of PD and other neurodegenerative diseases. In recent years several genes have been identified as key molecules in driving *Wallerian degeneration*, a form of axon degeneration whereby axons severed from their cell body undergo rapid fragmentation and clearance (Waller, 1850). Wallerian degeneration is an active cellular process that can be suppressed by expression or suppression of signalling molecules. Suppression of Wallerian degeneration was first demonstrated in mice expressing the neomorphic gene *Wallerian degeneration slow* (Wld^S), composed of Nmnat1 and the N-terminal of ubiquitination factor E4B (Coleman et al., 1998; Lunn et al., 1989; Perry et al., 1990). Expression of Wld^S has been previously shown to suppress some forms of axon destruction in several models of neurodegeneration (Fischer et al., 2005; Meyer zu Horste et al., 2011; Mi et al., 2005; Wang et al., 2002; Wang et al., 2001) including toxin induced parkinsonism (Antenor-Dorsey and O'Malley, 2012; Cheng and Burke, 2010; Hasbani and O'Malley, 2006; Sajadi et al., 2004). Despite highlighting the existence of a programmed mechanism for axon destruction and showing promising disease modifying activity, mimicking the neomorphic action Wld^S has proven challenging. In an effort to identify endogenous genes driving programmed axon destruction, we previously conducted an unbiased forward genetic screen in *Drosophila*, identifying Sarm1 as a potent driver of severed axon destruction (Osterloh et al., 2012). Sarm1 promotes axon destruction by actively hydrolysing NAD+, which has been proposed to deplete severed axons of this critical bioenergetic molecule (Essuman et al., 2017; Gerdts et al., 2015; Neukomm et al., 2017; Summers et al., 2016). The neuron autonomous role for Sarm1 in Wallerian axon destruction is conserved in mammals (Gerdts et al., 2013; Osterloh et al., 2012), making Sarm1 a promising therapeutic candidate for supressing axon destruction in neurodegenerative disease and injuries of the nervous system. *In vivo* experiments have however suggested Sarm1 has roles in some, but not all forms of axon degeneration. Sarm1 contributes to optic nerve crush models of glaucoma (Fernandes et al., 2018), traumatic brain injury (Henninger et al., 2016) and both chemotherapy- and metabolic dysfunction-induced peripheral neuropathy (Turkiew et al., 2017). In contrast Sarm1 does not appear to contribute to progressive degeneration of motor neurons seen in genetic models of amyotrophic lateral sclerosis (Peters et al., 2018; Sreedharan et al., 2015).

In the present study we aimed to define if loss of Sarm1 can supress axon destruction in PD. Primary neuron tissue culture studies have demonstrated that Sarm1 contributes to the destruction of axons in response to toxins which mimic some of the mitochondrial dysfunction associated degenerative processes seen in PD (Summers et al., 2014). To determine whether Sarm1 contributes to *in vivo* dopaminergic axon destruction, we have tested whether Sarm1 drives the destruction of dopaminergic neurons in toxin and viral-mediated alpha-synuclein overexpression mouse models of parkinsonism. While Sarm1 knockout potently suppressed 6-OHDA lesioning of the medial forebrain bundle, loss of Sarm1 did not block axon dying back after 6-OHDA treatment of DA neuron terminals nor AAV-mediated alpha-synuclein overexpression models of PD. Our data support the notion that existing PD models induce genetically distinct programs of axon degeneration, some of which are Sarm1-dependent.

2. Materials and methods

2.1. Animals

Sarm1 mice were obtained from Jackson laboratories (stock #018069) and maintained on a C57Bl6/J background. Experimental cohorts of wild type, Sarm1 heterozygous and Sarm1 homozygous null mice were generated through crossing of heterozygous animals. Mice were maintained on a 12 h light/dark cycle, with *ad libitum* access to standard mouse chow and water. Experiments were conducted by a researchers blinded to genotype. All experimental protocols were approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee.

2.2. Surgery

3-Month old male mice were anesthetized via inhalation of gaseous isoflurane, placed in a stereotaxic frame, the scalp shaved and cleaned, and a small anterior-posterior incision made to expose the skull. The injection site was identified and a small bore drilled using a dental drill bit. A Hamilton Neuros syringe fitted with a 33 gauge needle was used to deliver either 6-OHDA or AAV vector, delivered at a flow rate of 0.25 μl/min using an automated injection pump and control unit (World Precision Instruments). Coordinates for unilateral lesioning of the medial forebrain bundle were AP −1.2, ML +1.2, DV −4.7, with a single delivery of 750 μl of 6.5 μg/μl 6-OHDA freebase dissolved in sterile saline containing 0.02% ascorbic acid. For unilateral lesioning of the striatum, two injections were given, at coordinates AP + 1.0, ML + 2.1, DV 2.9 and AP +0.3, ML + 2.3, DV 2.9, with each injection site receiving 750 μl of 6.5 μg/μl 6-OHDA free base dissolved in sterile saline containing 0.02% ascorbic acid. For delivery of AAV6-SNCA, vectors were unilaterally delivered to the substantia nigra pars compacta at coordinate AP −2.8, ML 1.2, DV 3.8, delivering 1 μl 1.3×10^{13} vg/μl. Post-injection, the needle was left in place for 1 min to allow diffusion of injected solution, followed by slow withdrawal of the needle. The skin of the scalp was then sutured, the animal treated with analgesic medication and allowed to recover in heated home cage. Post-surgery animals were housed separately.

2.3. Amphetamine induced rotational behaviour

At 7-days post-MFB lesion, mice were treated with 2.5 mg/kg amphetamine via intraperitoneal injection to induced rotational behaviour. Following the injection, mice were immediately placed in a glass beaker cage. Rotational behaviour was recorded over a 60-min test period and number of complete contralateral and ipsilateral rotations per 5-min bin counted.

2.4. Alpha-Synuclein expression vectors

A custom gene block was synthesised containing wild type human *SNCA*, a 5′ alpha subunit of Woodchuck hepatitis virus post-transcriptional regulatory element (*WPRE*) and SV40 polyA tail (Integrated DNA Technologies). The *SNCA* construct was sub-cloned into an AAV backbone with human Synapsin 1 promotor mediated neuron

restricted gene expression (pTR-hSYN1-GFP, Gift from Miguel Sena-Esteves, University of Massachusetts Medical School (Vodicka et al., 2016)). Packaging of the plasmids into AAV6 was performed by the University of Massachusetts Medical School virus core facility.

2.5. Histology

For harvesting of brains for histological analysis, mice were perfused with a pre-wash of PBS, followed by 4% paraformaldehyde dissolved in PBS. The brain was removed and post-fixed overnight in 4% paraformaldehyde dissolved in PBS, followed by sinking in 25% sucrose and storage at 4C. 40 μm horizontal sections were collected using a Leica VT1200S vibratome and stored in an antifreeze solution (30% Glycerol, 30% ethoxyethanol, 40% PBS) at −20 °C. Free-floating sections were washed in PBS, then incubated in a blocking solution of 10% donkey serum (ThermoFisher) in PBS containing 0.4% TritonX_{100}. Primary antibodies were diluted in the same blocking solution and incubated overnight at 4C. Following washing with PBS, sections were treated with AlexaFluor secondary antibodies (ThermoFisher) for 3 h at room temperature, followed by a final wash. Sections were mounted onto glass slides and allowed to dry overnight. Background auto-fluorescence was blocked by immersion of slides in a solution of 0.4% sudan black B in 70% ethanol, followed by wash in 50% ethanol. Glass coverslips were applied using ImmunMount (Shandon).

Images for analysis of dopaminergic terminal preservation following medial forebrain bundle lesion or treatment with SNCA vectors was conducted using a Zeiss AxioObserver three images were randomly collected at 40× magnification within the striata in a 1:6 section series, and distribution of tyrosine hydroxylase staining assessed using CellProfiler (Broad institute (Lamprecht et al., 2007)). For dopaminergic neuron survival and alpha-synuclein expression quantification, cell bodies staining positive for tyrosine hydroxylase or alpha-synuclein in a 1:3 (6-OHDA) or 1:6 (AAV-SNCA) section series, and quantified at 40× magnification by a researcher blinded to genotype. For analysis of dopaminergic terminal preservation following striatal lesioning, images were collected using Innovative Imaging Innovations (3I) spinning-disc confocal microscope. Three images were randomly collected within the striata in a 1:6 section series, and volume of TH staining was assessed using Velocity 3D image analysis software (Perkin Elmer). Total sampled tyrosine hydroxylase staining or cell number per hemisphere were determined and presented as percentage relative to the unlesioned hemisphere.

2.6. Antibodies

The following antibodies were used for immunohistochemical staining; Rabbit anti-Tyrosine Hydroxylase, PelFreeze (P40101-150) 1:1000; Mouse anti-Tyrosine Hydroxylase (TH-2), Sigma (T1299) 1:1000; Rabbit anti-alpha Synuclein, Life Technologies (701085) 1:1000; Rabbit anti-alpha Synuclein pSer129 (EP1536Y) AbCam (ab51253) 1:1000; Alexa Fluor 488 Donkey anti-Rabbit Life Technologies (A21206) 1:000 Alexa Fluor 546 Donkey anti-Rabbit Life Technologies (A10040) 1:1000; Alexa Fluor 488 Donkey anti-Mouse Life Technologies (A21202) 1:1000; Alexa Fluor 546 Donkey anti-Mouse Life Technologies (A10036) 1:1000.

2.7. High performance liquid chromatography

For harvesting tissues for HPLC analysis, animals were euthanized by overdose with CO_2 followed by cervical dislocation. Brains dissected followed by microdissection of intact and lesioned striata which were frozen in liquid nitrogen and stored at −80 °C. For HPLC analysis, individual striata were weighed, then homogenised in 150 mM phosphoric acid (Fisher) and 0.2 mM EDTA (Fisher). Sample were centrifuged at 4 °C for 15 min at 17000 RCF. Supernatant was filtered by centrifuge tube filters (Costar Spin-X Centrifuge Tube Filter cellulose acetate filter, 0.22 μm pore size) and stored at −80 °C. Dopamine, DOPAC and HVA concentration were analysed using standard reverse-phase HPLC with electrochemical detection, as described previously (Chen et al., 2001; Xu et al., 2006). Dopamine and its metabolite concentrations were determined as picomoles per milligram of wet striatal tissue and presented as percentage of ipsilateral (lesioned) striata relative to the contralateral (non-lesioned) control striata.

2.8. Statistics

Statistical analysis was conducted using GraphPad Prism software. One-way or two-way analysis of variance (ANOVA) with Bonferroni post-hoc analysis, unpaired *t*-tests or linear regression were applied to data as appropriate, with all data displayed as mean ± standard error of mean (Mean ± SEM).

2.9. Data availability

All data presented in this study can be made available upon reasonable request.

3. Results

3.1. Sarm1 contributes to the destruction of chemically lesioned dopaminergic axons

The mechanisms by which DA neurons degenerate in Parkinson's disease and related disorders represent a potential target for therapeutic intervention that is poorly defined. To explore if Sarm1-mediated axon destruction contributes to the degeneration of DA neurons in PD, we set out to test the established models of dopaminergic neuron degeneration in mice either heterozygous (Het) or null (KO) for the gene. As Sarm1 has been robustly demonstrated to play a critical role in the destruction of axons severed from their cell bodies (Gerdts et al., 2013; Osterloh et al., 2012; Peters et al., 2018), we first set out to establish whether it contributes to degeneration of dopaminergic axons following lesioning injuries. Neurons of the mouse nigrostriatal tract are an ideal target for *in vivo* study of DA neuron response to injury, with cell bodies of the SNpc projecting axons via the medial forebrain bundle (MFB) to distal synaptic targets located within the striatum, allowing axon injuries to be targeted to specific compartments of these cells.

To induce severing injuries of dopaminergic axons from their associated cell bodies we utilised a chemical lesioning approach, using stereotactic injection of 6-hydroxydopamine (6-OHDA) into defined regions of the brain to trigger selective atrophy and destruction of DA and noradrenergic neurons. We first aimed to determine whether Wallerian degeneration of DA axons and synapses separated from their cell bodies is promoted by Sarm1. Following lesioning injury we anticipated that distal DA axons would undergo typical Wallerian degeneration in mice expressing Sarm1 (ie WT and Sarm1 Het), with a rapid fragmentation of severed axons followed by clearance of debris by surrounding glia. Unilateral injection of 6-OHDA in the MFB was used to lesions DA axons midway between the SNpc cell bodies and their synaptic terminals in the striatum, which allowed us to quantify both degeneration of terminal and cells bodies, and compare this to the uninjured contralateral hemisphere (Fig. 1). Immunostaining for DA neuron specific tyrosine hydroxylase (TH) was used for detection and quantification of DA terminals within the striata of mice 3- and 7- days after 6-OHDA lesioning of the MFB (Fig. 1A-C). As anticipated in WT animals, DA terminals in the uninjured control hemisphere were abundant, however at 3-days post-lesion TH staining was undetectable in the lesioned hemisphere, indicating profound degeneration and clearance of severed terminals. In striking contrast, we found significant preservation of DA terminals in the lesioned hemispheres of Sarm1 KO, and unexpectedly also in Sarm1 Het mice, with over half the striatal DA terminals remaining. At day 7 post-lesion, significant numbers of DA terminals remained in KO mice,

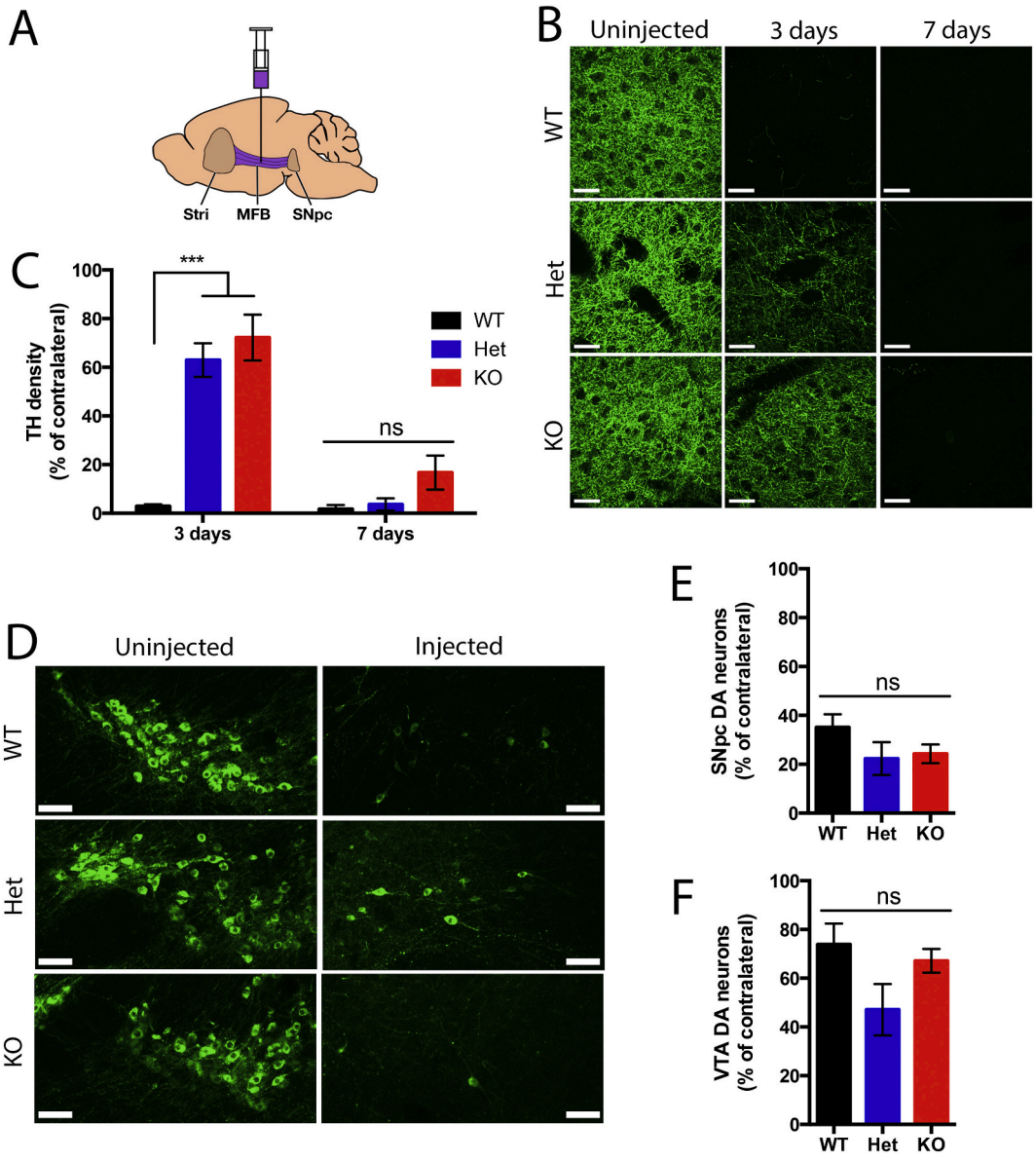

Fig. 1. Protection of lesioned dopaminergic axons in Sarm1 null mice. (A) Schematic showing MFB lesioning model indicating target region for 6-OHDA injection. (B) Tyrosine hydroxylase (TH) staining of DA terminals in the striatum of Sarm1 null mice, 3 and 7 days post 6-OHDA lesion of the MFB. (C) Quantification of striatal TH density ($n = 4–8$, 2-way ANOVA, Bonferonni post-hoc test *** = $p < 0.001$). (D) Horizontal section containing SNpc DA neuron cell bodies immunostained for TH 14 days post MFB lesion. (D,E) Quantification of DA neuron number in the SNpc (E) and VTA (F) 14 days post MFB, expressed as percentage of dopaminergic neuron cell bodies in the lesioned hemisphere relative to the uninjected contralateral hemisphere (n = 4–5, one-way ANOVA). Scale bars A = 30 μm, C = 50 μm.

though they were absent in WT and negligible in Sarm1 heterozygous animals (Two-way ANOVA with Bonferroni multiple comparisons test; $n = 4–8$; Genotype F (2,33) = 22.74, $p < 0.0001$; Timepoint F (1,33) = 58.35, $p < 0.0001$; Interaction F (2, 33) = 13.05, $p < 0.0001$WT vs Het $P < 0.0001$; WT vs KO $p < 0.0001$).

Following 6-OHDA lesioning DA cell bodies of the SNpc and to a lesser extent the adjacent ventral tegmental area (VTA) are also lost. To assess whether Sarm1 contributes to the death of DA neurons we quantified the number of cell bodies present in the lesioned and control SNpc and VTA proximal to the 6-OHDA lesioning injury (Fig. 1D). To allow for sufficient time to induce neuronal cell body degeneration animals were assessed at 14-days post-lesion. We observed comparable rates of cell death of DA cell bodies in the SNpc (Fig. 1E) and ventral tegmental area (Fig. 1F) in control and Sarm1 mutant mice 14-days post-lesion.

Having demonstrated Sarm1 contributes to the physical destruction of DA terminals in mice, we next assessed whether remaining synapses retained the ability to maintain stored reserves of vesicular DA or its metabolites. Using high performance liquid chromatography (HPLC) we quantified levels of striatal DA, DOPAC and HVA at 3-, 7- and 14-days post 6-OHDA lesion relative to the intact contralateral hemisphere (Fig. 2). Whilst stored DA was absent in lesioned hemisphere of wild type mice, (Fig. 2A) significant protection was observed in Sarm1 KO animals in both 3- and 7-day time points post-lesion, with a non-significant trend also observed in heterozygotes (Two-way ANOVA with Bonferroni multiple comparisons test, $n = 3–4$; Genotype F (2, 25) = 19.57 $P < 0.0001$; Timepoint F (2, 25) = 22.03, $P < 0.0001$; Interaction F (4, 25) = 6.405, $P = 0.0011$, 3-days WT vs KO $p < 0.0001$, 7-days WT vs KO $p < 0.05$). DOPAC was detected in the lesioned hemisphere of all animals at 3-days (Fig. 2B), with a significant elevation seen in heterozygous animals relative to the intact hemisphere (Two-way ANOVA with Bonferroni multiple comparisons test, n = 3–4; Genotype F

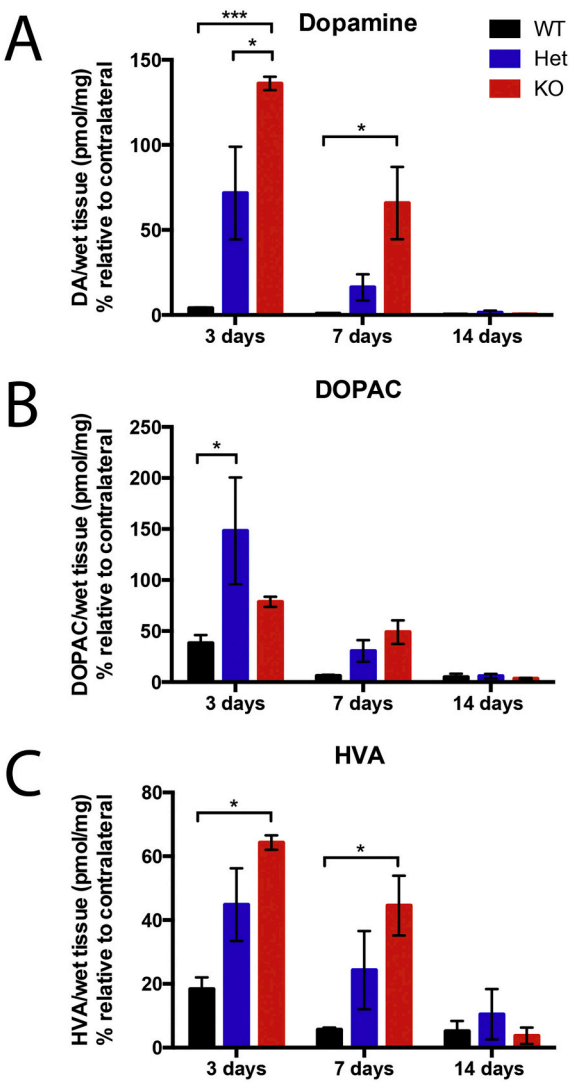

Fig. 2. Significant presence of dopamine and its metabolites in the striata of MFB 6-OHDA lesioned Sarm1 knock out mice. High-Performance Liquid Chromatography (HPLC) quantification of striatal (A) dopamine, (B) DOPAC and (C) HVA at 3-, 7- and 14-days post 6-OHDA lesion of the MFB in WT and Sarm1 knock out mice (mean ± SEM, $n = 3$–4, 2-way ANOVA, Bonferonni post-hoc, ** $p < 0.01$, *** $p < 0.001$).

(2, 25) = 3.774, $P = 0.0370$; Timepoint F (2, 25) = 13.78, $P < 0.0001$; 3-days WT vs Het $p < 0.05$). DOPAC was not preserved at 7-days post-lesion in either Het and KO animals. Significant protection of HVA (Fig. 2C) levels was seen in lesioned Sarm1 KO striata at both 3- and 7-days post-lesion, with a non-significant trend toward protection also observed in Hets (Genotype F (2, 25) = 9.594, $P = 0.0008$; Timepoint F (2, 25) = 16.07, $P < 0.0001$; 3-days WT vs KO $p < 0.05$, 7-days WT vs KO $p < 0.05$). No significant protection of DA nor its metabolites was detected at 14-days post-lesion in any of the experimental cohorts.

The presence of DA in lesioned striata of Sarm1 mutant mice suggests that blocking Wallerian degeneration allows severed terminals to retain at least the functional capacity to store neurotransmitters. We next set out to test whether these severed terminals retained the ability to signal to post-synaptic neurons of the striata, utilising d-amphetamine (dAMPH), which potently stimulates the release of dopamine and induces asymmetric rotational behaviour in mice harbouring unilateral 6-OHDA lesions. Typically, after clearance of lesioned DA terminals, wild type mice will undergo rotational behaviour ipsilateral of the lesion (i.e. in the direction of the lesioned hemisphere). Indeed, at 7-days post-MFB lesion we found control WT mice, where nigrostriatal terminals and DA are absent, underwent sustained ipsilateral rotational behaviour following stimulation with dAMPH (Fig. 3). However, in striking contrast, following dAMPH stimulation Sarm1 KO, and to a comparable levels in Sarm1 Het mice, demonstrated sustained rotations in the direction contralateral to the lesion (Two-way ANOVA with Bonferroni multiple comparisons test, $n = 4$–6; Genotype F (2, 143) = 58.76 $P <$ 0.0001; Time F (11, 143) = 1.731 $P = 0.0722$; Interaction F (22, 143) = 0.8549 $P = 0.6530$; post-hoc annotation Fig. 3). This non-typical rotational behaviour is in agreement with a previous report of Wld^S mice following MFB lesioning, where dopaminergic terminals were also found to be preserved (Sajadi et al., 2004). The pronounced contralateral rotations observed in both Wld^S transgenic and Sarm1 mutant mice suggests that following severing injuries rather than remaining inactive, nigrostriatal terminals may continue to synthesise and accumulate dopamine, which stimulated through non-physiological dAMPH-induced DA release elicits an enhanced response in the contralateral direction.

3.2. *Sarm1 does not contribute to retrograde degeneration of dopaminergic axons*

Lesioning of the MFB demonstrated that the synaptic terminals of DA neurons lacking Sarm1 are both morphologically and functionally protected from Wallerian degeneration following severing injuries. Breakage of the axon along its length, degeneration and synaptic loss in DA neuron populations may occur during neurodegeneration in PD, however transecting injuries are not likely the major driver of neurodegeneration in PD and related "dying back" disorders. Indeed, evidence from PD patients suggests DA neurons undergo such a dying back process, where degeneration initiates at distal synapses and progresses to the cell bodies (Tagliaferro and Burke, 2016). To determine whether Sarm1 contributes to axonal dying back of SNpc DA neurons more typical of PD, we conducted unilateral 6-OHDA lesioning of DA neurites and synaptic terminals within the striatum (Fig. 4). TH immunostaining for DA axon terminals within the lesioned striata of both WT control and Sarm1 KO animals detected only the sparse presence of fragmented DA terminals, with quantification revealing no significant protection in mice lacking Sarm1 (Fig. 4A, B). As 6-OHDA lesioning is an acute chemical insult, it is feasible that any contribution of Sarm1 in die back degeneration of distal portions of these DA axons within the striatum may be masked by the severity of the injury. To assess whether Sarm1

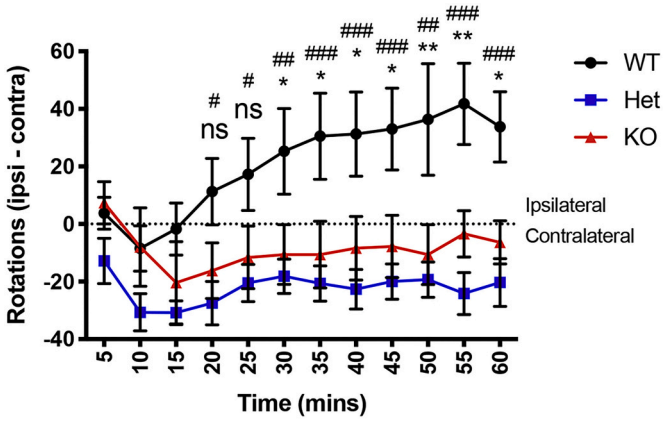

Fig. 3. Amphetamine induced rotational behaviour of mice lacking Sarm1 shows preservation of dopamine release function. Rotational behaviour of WT and Sarm1 KO mice administered with 2.5 mg/kg dAMPH 7 days post MFB 6-OHDA, recorded over a 60 min test period in 5-min bins. (Mean ± SEM, n = 4–6, 2-way ANOVA, Bonferroni post-hoc, * $p < 0.05$, ** $p < 0.01$, *** $p <$ 0.001, # = WT vs Het, * = WT vs KO, Het vs KO showed no significant differences).

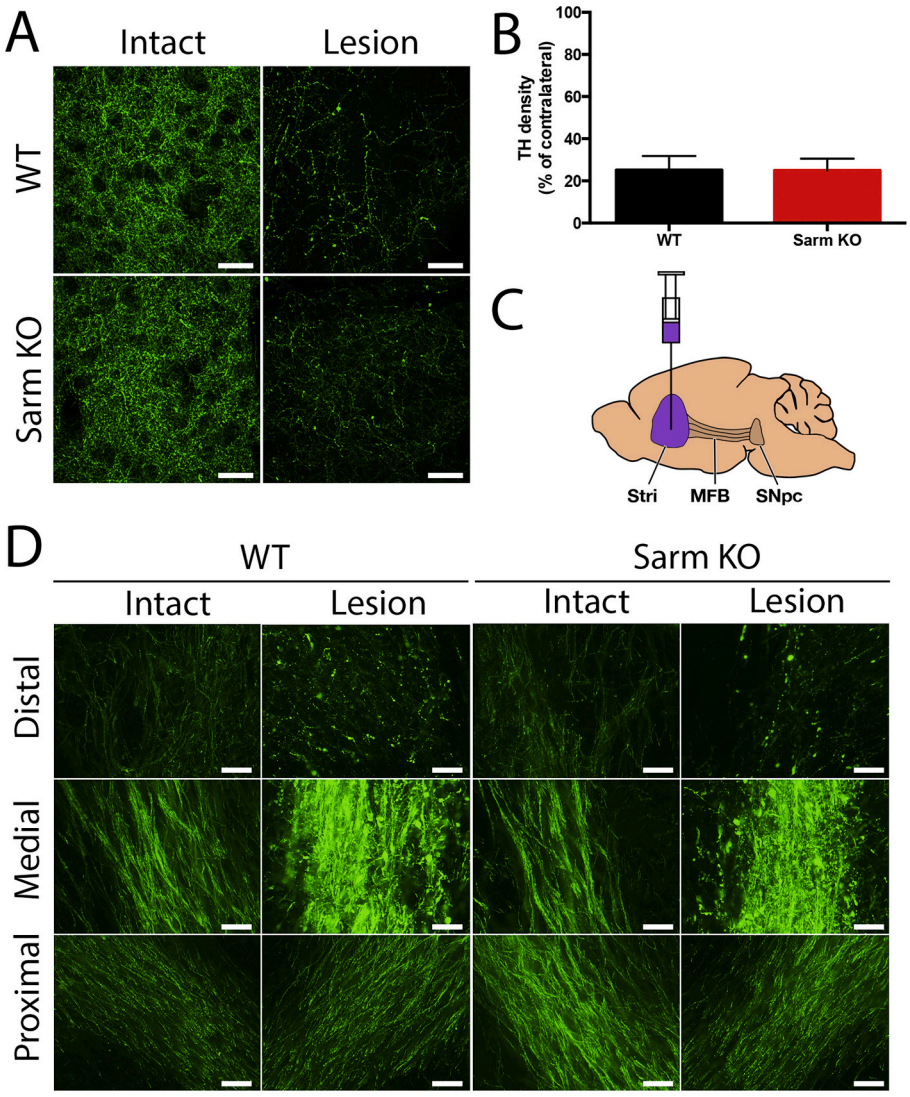

Fig. 4. Sarm1 does not contribute to retrograde degeneration of 6-OHDA lesioned dopaminergic axons. (A) TH staining of the striatum of WT mouse 3 days post striatal lesion TH staining was significantly reduced with only degenerating portions of axons remaining. (B) Quantification of TH staining of 3 day post-lesioned WT and Sarm1 KO mice revealed no preservation of terminals relative to the unlesioned contralateral control hemisphere (mean ± SEM, n = 3, unpaired *t*-test, p = ns). (C) Schematic showing striatal lesioning model indicating target region for 6-OHDA injection. (D) Micrographs showing TH immunostaining of the distal, medial and proximal portions of the MFB (relative to SNpc) 3 days post lesioning of the striatum. Significant degeneration is seen in the distal and medial portions in both WT and Sarm1 KO mice indicated by the presence of large swollen, dystrophic axons, though the proximal regions are more intact. Scale bars A = 30 μm, D = 60 μm).

contributes to die back of these axons beyond their striatal portion, we next examined DA axons within the MFB of WT control and Sarm1 KO animals 3-days post-lesion (Fig. 4D). In both control and Sarm1 KO animals, a consistent pattern of DA axon degeneration was evident within the MFB of the lesioned hemisphere. In both genotypes swollen and dystrophic neurites were observed in distal and medial axon portions but not proximal regions adjacent to SNpc cell bodies. Taken together this data suggests striatal 6-OHDA lesioning triggers a process of DA axon die back that progresses beyond the striatum, however did not suggest a contribution for Sarm1 in this degeneration.

Though 6-OHDA lesioning mimics aspects of parkinsonian neurodegeneration by selectively injuring dopaminergic neurons, it is a rapidly destructive, acute insult that does not recapitulate the more slowly progressive degeneration seen in patients. In an effort to assess the role of Sarm1 in a more physiological, slowly progressing model of PD, we next turned to adeno-associated virus (AAV) mediated expression of *SNCA*, an approach which has previously been utilised to induce Parkinsonian degeneration of DA neurons (Decressac et al., 2012), with increased expression of *SNCA* likely representing a more physiologically relevant (Chartier-Harlin et al., 2004; Ibáñez et al., 2004; Singleton et al., 2003) model of axon degeneration. We generated adeno-associated viral (AAV6) vectors to drive expression of human wild type SNCA by the neuron specific human synapsin 1 promoter. AAV6-SNCA were injected unilaterally into the SNpc of 10–12 week old WT, Sarm1 Het and KO mice with the non-injected contralateral hemisphere serving as an internal control (Fig. 5). Immunostaining for alpha-synuclein revealed widespread expression throughout SNpc neurons and their striatal terminals in the brains of injected mice 12-weeks post-surgery, with evidence of histopathological hallmarks of synuclein associated neuron damage including swollen neurites containing alpha synuclein (Fig. 5A) and its disease-associated (Fujiwara et al., 2002) Ser^{129} phosphorylated species (Fig. 5B).

To assess if our model of alpha synuclein overexpression impacted axon survival, experimental cohorts were aged for 6-months post-surgery, followed by collection of brains for immunohistological assessment. To define if increased expression of alpha synuclein induced axonal die back we immunostained striata for presence of TH positive neurites, observing a notable reduction in dopaminergic neurites present in the striata of injected hemisphere of WT control and Sarm1 mutant mice (Fig. 5D, E). Quantification and statistical analysis of TH immunostaining indeed revealed a significant effect of treatment with AAV6-SNCA, however no significant difference was observed among controls and Sarm1 knockouts, indicating Sarm1 indicating that loss of Sarm1 alone is not sufficient to block alpha-synuclein mediated degeneration of dopaminergic terminals (Two-way ANOVA with Bonferroni multiple comparisons test, $n = 3$–8; Treatment F (1, 33) = 29.61, $P < 0.0001$; Genotype F (2, 33) = 0.2364, $P = 0.7908$; Interaction F (2, 33) = 0.6026 $P = 0.5533$). Neurodegeneration in PD is perhaps best defined

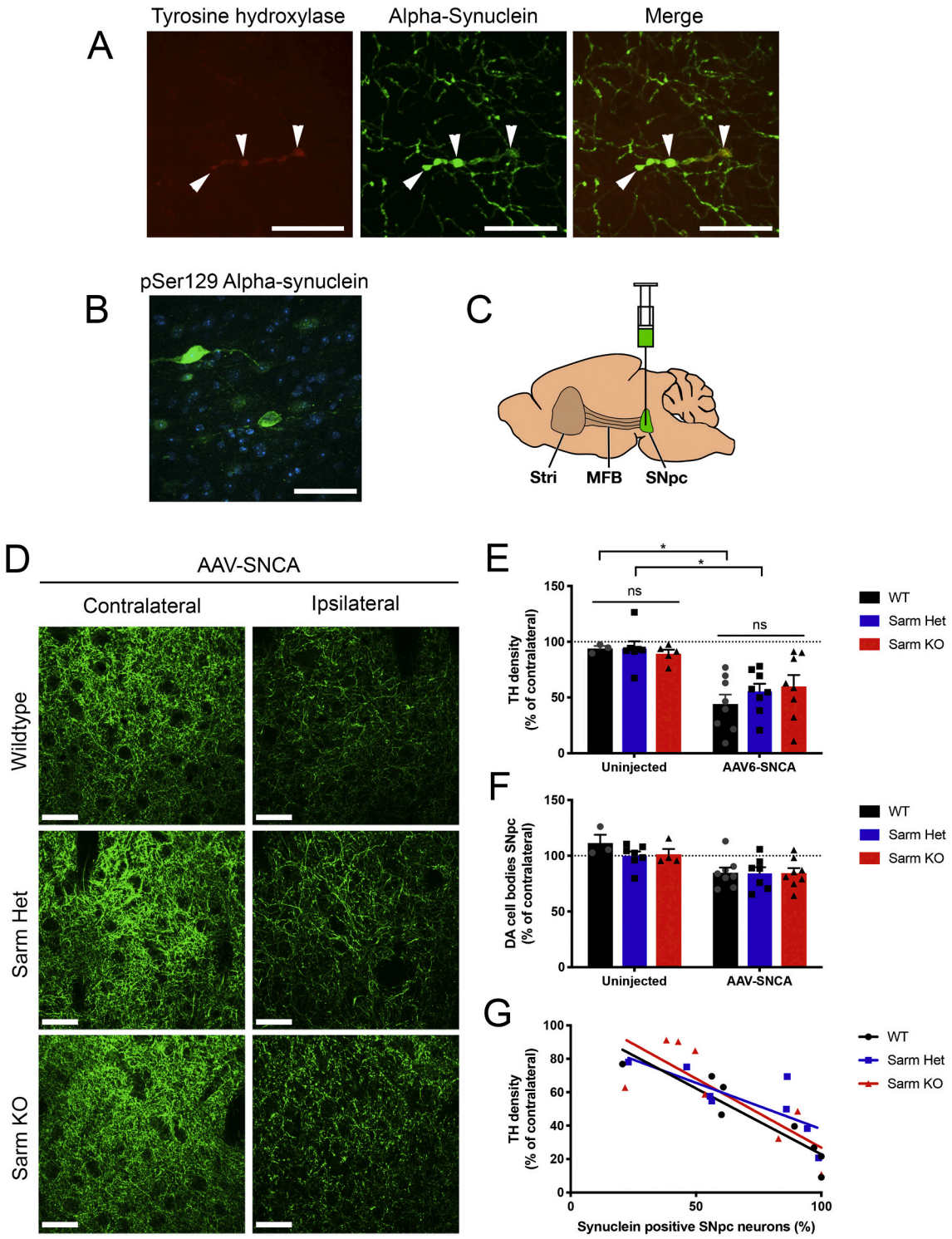

Fig. 5. Sarm1 does not contribute to alpha synuclein-induced degeneration of dopaminergic axons. (A) Human alpha-synuclein and (B) Ser129-phosophylated species were detected within beaded DA axons in the striatum of 12-week old injected wild type mice. (C) Schematic showing AAV6-SNCA model targeting dopaminergic neurons of the SNpc. (D) TH staining of DA axons in contralateral (uninjected hemisphere) and ipsilateral striatum (injected hemisphere) of WT and Sarm1 null mice 6-months post-injection. (E) Quantification of striatal TH staining density in AAV6-SNCA injected mice compared to uninjected control mice (mean ± SEM two-way ANOVA, * $p < 0.05$). (F) Quantification of SNpc DA cell bodies relative to the contralateral hemisphere of AAV-SNCA injected groups compared to uninjected animals (mean ± SEM, two-way ANOVA, n = 3–8). (G) Striatal TH density correlated with number of alpha-synuclein positive neurons in the transfected SNpc (Linear regression; slope p = ns, intercept p = ns). Scale bar A,B = 20 μm; C = 60 μm.

by the dramatic loss of DA neuron cell bodies in the SNpc. To assess whether Sarm1 contributes to this degeneration we quantified SNpc DA cell bodies in WT and Sarm1 mutant mice at the 6-month post-infection timepoint. Quantification revealed a modest loss of DA neuron cell bodies in the SNCA expressing hemisphere of all genetic backgrounds, indicated by a significant effect of treatment group, however *post-hoc* analysis failed to detect any significant difference in cell count between AAV6-SNCA and uninjected control con hemispheres. No significant

differences were detected between WT or Sarm1 mutant genotypes, again indicating that loss of Sarm1 alone does not block degeneration of DA cell bodies (Fig. 5F, Two-way ANOVA with Bonferroni multiple comparisons test, n = 3–8; Treatment F (1,31) = 19.84, $P = 0.0001$; Genotype F (2,31) = 0.6554, $P = 0.5263$; Interaction F (2, 31) = 0.5699, $P = 0.5714$). To account for variability in transfection efficiency which may obscure more subtle changes in DA neuron degeneration, we finally quantified the number of *SNCA* transfected DA cell bodies and correlated this with loss of TH positive terminals for individual mice (Fig. 5G), however we again failed to detect any significant differences between WT and Sarm1 mutant cohorts. Taken together these data suggest that whilst Sarm1-mediated axon destruction drives the Wallerian degeneration of DA axons severed from their cell bodies following lesioning injuries, it is not the primary driver of PD relevant alpha-synuclein induced degeneration of SNpc dopaminergic terminals and cell bodies, though it remains feasible it may contribute in parallel to other pathways for axon destruction.

4. Discussion

Axon degeneration can be triggered through a variety of pathogenic insults, ranging from physical injury, autoimmune damage and disease-associated neurodegeneration such as that seen in Parkinson's disease. Whilst it was initially speculated that axon degeneration in development and many diseases may be mechanistically related (Raff et al., 2002), emerging experimental evidence is increasingly supporting the opposite notion—that different forms of injury or trauma activate diverse genetic pathways driving the destruction of axons. Indeed, our study further supports the notion of diverse genetic mechanisms of axon destruction being engaged even in the very same neurons when severed by 6-OHDA compared to application at DA terminals. Reduced expression of Sarm1 potently delayed the degeneration of dopaminergic axons distal to a chemical lesion in the MFB, which were remarkably capable of maintaining the ability to both store and release dopamine several days post injury. However, retrograde die back destruction of the same DA neuron axons progressed unhindered following chemical lesioning of terminals in animals lacking Sarm1. Similarly, loss of Sarm1 did not alter loss of terminals in our model of PD-associated alpha-synuclein overexpression. Thus despite being essential for efficient destruction of lesioned dopaminergic axons, Sarm1 alone does not appear to drive the process of axon die back or synuclein overexpression induced damage to these neurons. This does not exclude a role for Sarm1—for instance, Sarm1 could be working with other redundant genetic pathways to drive destruction in the die back models, which remain to identified. Nevertheless, our alpha synuclein data strongly suggests that classical, Sarm1-mediated Wallerian degeneration is not the major driving force contributing to axon destruction in PD and associated synucleinopathies.

PD is a genetically heterogenous condition, with *SNCA* mutations accounting only for a small fraction of familial cases (Chartier-Harlin et al., 2004; Ibáñez et al., 2004; Polymeropoulos et al., 1997; Singleton et al., 2003). Our rodent model of synuclein overexpression provides further support to the notion that axons undergo a process of retrograde destruction in PD, with loss of striatal terminals preceding any detectable loss of neuron cell bodies in the SNpc. As more PD associated genes are identified, we will gain a greater depth of understanding of the cellular processes driving disease onset and progression. In the various subtypes of familial PD, and even more so in the more common idiopathic form of disease, neither the precise pathogenic events nor the processes by which axons degenerate are fully understood. For example, Sarm1 has previously been shown to protect *in vitro* rodent sensory neurons from mitochondria-depolarisation induced death (Summers et al., 2014). As mutations in key mitophagy genes *PINK1* (Kitada et al., 1998; Matsumine et al., 1997) and *PRKN* are causative of PD (Larsen et al., 2018), it is feasible Sarm1 may contribute to the specific forms of axon destruction induced by mitochondria dysfunction. Other PD-associated genes including *LRRK2* (Paisán-Ruíz et al., 2004; Zimprich et al., 2004) and *GBA* (Sidransky et al., 2009) have been linked to dysfunction of the endo-lysosomal system, for which roles for Wallerian degeneration associated genes have yet to be explored. In order to gain a better understanding of if and when Sarm1 inactivation may be of therapeutic benefit in PD, it will be essential to explore a broad range of disease-associated models of DA axon destruction.

In parallel to the continued identification of genes associated with PD, the repertoire found to regulate Wallerian degeneration is also expanding (Coleman et al., 1998; Farley et al., 2018; Mack et al., 2001; Miller et al., 2009; Neukomm et al., 2017; Osterloh et al., 2012; Xiong et al., 2012; Yang et al., 2015). Whilst loss of Sarm1 potently inhibits destruction of severed axons but has varied effect in models of neurodegeneration (Fernandes et al., 2018; Peters et al., 2018; Turkiew et al., 2017), the role of other axon death genes including *Highwire*, *Axundead*, *Nmnat* and the MAPK signalling cascade remain unclear. Functional redundancy may exist in axon degeneration pathways, and its blockade may require inhibition of multiple pathways simultaneously to save axons. Further characterization of Wallerian degeneration signalling pathways, and the mechanisms of programmed axon destruction in disease will be essential to bridge this gap in our understanding and may help identify meaningful therapeutic approaches to neurodegenerative disease.

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Competing Interests

Marc Freeman is a co-founder of Nura Bio.

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