

Research paper

Rab7 reduces α -synuclein toxicity in rats and primary neurons

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ABSTRACT

During the pathogenesis of Parkinson's disease (PD), aggregation of alpha-synuclein (α Syn) induces a vicious cycle of cellular impairments that lead to neurodegeneration. Consequently, removing toxic α Syn aggregates constitutes a plausible strategy against PD. In this work, we tested whether stimulating the autolysosomal degradation of α Syn aggregates through the Ras-related in brain 7 (Rab7) pathway can reverse α Syn-induced cellular impairment and prevent neurodegeneration in vivo. The disease-related A53T mutant of α Syn was expressed in primary neurons and in dopaminergic neurons of the rat brain simultaneously with wild type (WT) Rab7 or the T22N mutant as negative control. The cellular integrity was quantified by morphological and biochemical analyses. In primary neurons, WT Rab7 rescued the α Syn-induced loss of neurons and neurites. Furthermore, Rab7 decreased the amount of reactive oxygen species and the amount of Triton X-100 insoluble α Syn. In rat brain, WT Rab7 reduced α Syn-induced loss of dopaminergic axon terminals in the striatum and the loss of dopaminergic dendrites in the substantia nigra pars reticulata. Further, WT Rab7 lowered α Syn pathology as quantified by phosphorylated α Syn staining. Finally, WT Rab7 attenuated α Syn-induced DNA damage in primary neurons and rat brain. In brief, Rab7 reduced α Syn-induced pathology, ameliorated α Syn-induced neuronal degeneration, oxidative stress and DNA damage. These findings indicate that Rab7 is able to disrupt the vicious cycle of cellular impairment, α Syn pathology and neurodegeneration present in PD. Stimulation of Rab7 and the autolysosomal degradation pathway could therefore constitute a beneficial strategy for PD.

List of abbreviations

53BP1	tumor suppressor p53-binding protein 1
DAB	3,3-diaminobenzidine
DAT	dopamine transporter
DCFH-DA	dichlorodihydrofluorescein diacetate
DIV	days in vitro
DLB	dementia with Lewy bodies
GC	genome copy
GFP	green fluorescent protein
JC-1	tetraethylbenzimidazolylcarbocyanine iodide
LDH	lactate dehydrogenase
LN	Lewy neurites

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MSA	multiple system atrophy
PD	Parkinson's disease
phospho- α Syn	phosphorylated alpha-synuclein
PSD95	postsynaptic density protein 95
rAAV	recombinant adeno-associated viral vector
Rab7	Ras-related in brain 7
ROS	reactive oxygen species
SN	substantia nigra
SNC	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
TBS	Tris-buffered saline
TH	tyrosine hydroxylase

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VMAT2	vesicular monoamine transporter 2
WT	wild type
α Syn	alpha-synuclein

1. Introduction

Aggregation and accumulation of alpha-synuclein (α Syn) are key events in Parkinson's disease (PD) and other synucleinopathies. α Syn monomers are mainly located at presynaptic vesicles whereas misfolded α Syn oligomers and high molecular weight species can be found throughout the entire neuron. α Syn pathology affects diverse cellular functions, including: increasing reactive oxygen species (ROS), impaired mitochondrial function (Junn and Mouradian, 2002; Szegő et al., 2019), reduced proteasomal degradation and autophagy (Suzuki et al., 2020; Winslow and Rubinshtein, 2011) as well as DNA damage (Wang et al., 2016b). However, the pathogenesis of PD does not follow a linear succession of events and is rather characterized by self-enhancing mechanisms. ROS and impaired mitochondrial function also increase α Syn pathology (Goodwin et al., 2013; Scudamore and Ciossek, 2018), as does impaired protein quality control and clearance (Klucken et al., 2012; Minakaki et al., 2018; Winslow and Rubinshtein, 2011). Moreover, autophagy can protect against oxidative stress (Galati et al., 2019; Guo et al., 2013; Kiffin et al., 2006) while oxidative stress can impair autophagy (Burgoyne, 2018; Frudd et al., 2018). DNA damage can also result from ROS imbalance (Milanese et al., 2018; Paiva et al., 2017), and it can induce autophagy (Eapen et al., 2017). Taken together, toxic α Syn species initiate a vicious circle of protein aggregation and cellular dysfunction. Removing toxic α Syn species has the potential to disrupt this toxic feedback loop, improve neuronal health and slow disease progression in synucleinopathies.

Aggregated α Syn is degraded via the autophago-lysosomal pathway. Mutations in genes encoding autophago-lysosomal pathway proteins, such as glucocerebrosidase (GBA), ATPase cation transporting 13A2 (ATP13A2), Ras-related in brain 39B (Rab39B), ADP-ribosylation factor-like protein 8B (Arl8b), lysine acetyltransferase 8 (KAT8), Ras-related in brain guanine nucleotide exchange factor 1 (RabGEF1) or vacuolar protein sorting ortholog 35 (VPS35), cause impairment of degradation, and are risk factors for PD (Bandres-Ciga et al., 2019; Chang et al., 2017; Gao et al., 2018; Sidransky et al., 2009; Verstraeten et al., 2015). The multi-step process of autophagy is regulated by several critical proteins, including the Ras-related in brain (Rab) family of small GTPases. Ras-related in brain 7 (Rab7) orchestrates trafficking of autophagosomes and late endosomes, catalyzes the fusion of autophagosomes with lysosomes, and regulates late maturation of autophagosomes. Rab7 malfunction hampers autolysosome formation, leads to the accumulation of dysfunctional autophagosomes and consequently impairs protein degradation (Baba et al., 2019; Kjos et al., 2017; Kuchitsu and Fukuda, 2018; Zhan et al., 2017). We previously demonstrated that Rab7 is the rate-limiting factor for α Syn aggregate clearance; its overexpression facilitates the removal of α Syn inclusions in human cells and alleviates α Syn-induced motor phenotype in fruit flies (Dinter et al., 2016).

In the present work, we investigated whether mammalian neurons can be protected against α Syn toxicity by Rab7-induced stimulation of aggregate clearance. Expression of α Syn carrying the disease-related A53T mutation was achieved in mouse primary neurons and in dopaminergic neurons of rat substantia nigra (SN) by a recombinant adeno-associated viral vector 2/7 (rAAV) as previously described (Karpinar et al., 2009; Van der Perren et al., 2015; Van Der Perren et al., 2011). Wild-type (WT) Rab7 was also expressed using rAAV. As a negative control, we used the Rab7 mutant T22N, which binds Rab7 effectors but not guanosine-5'-triphosphate (GTP) (Spinosa et al., 2008). First, we assessed the effect of Rab7 on α Syn pathology, α Syn-induced DNA damage, oxidative stress and cell death in mouse primary neurons.

Second, we analyzed the integrity of the dopaminergic nigrostriatal neurons in vivo. Finally, we investigated α Syn accumulation and DNA damage in the dopaminergic neurons. We demonstrate that inducing Rab7-mediated clearance of α Syn aggregates in neurons improves cellular integrity and reduces neurodegeneration.

2. Materials and methods

Source of chemicals, antibodies, composition of buffers, equipment and software used in this study are listed in Supplemental Table S1.

2.1. Recombinant AAV production and purification

Vector production and purification was carried out as previously described (Van Der Perren et al., 2011). The plasmids include: the constructs for the AAV7 serotype, the AAV transfer plasmid encoding either the human A53T mutant α Syn, the enhanced green fluorescent protein (GFP), human WT Rab7 with a double N-terminal HA-tag or T22N Rab7 with a double N-terminal HA-tag, under the control of the CMVie enhanced synapsin1 promoter and the pAdvDeltaF6 adenoviral helper plasmid. Real-time PCR analysis was used for genomic copy (GC) determination.

2.2. Primary neuronal culture

Primary cortical cultures were prepared from P1-3 C57BL6/J mouse pups of mixed sex as previously described (Szegő et al., 2019). Briefly, after dissection and trypsinization, dissociated neurons were plated onto poly-L-ornithine coated glass coverslip (100,000 cells per well in 24-well dishes), and maintained in Neurobasal A medium containing 2% B27, 0.5 mM glutamax and antibiotics. One third of the medium was changed on every third day, and from the second medium change on, no antibiotics were added. On DIV 12, neurons were transduced with one of the following combinations of rAAV vectors: (1) GFP, (2) GFP with T22N Rab7A, (3) GFP with WT Rab7A, (4) A53T α Syn with GFP, (5) A53T α Syn with T22N Rab7A or (6) A53T α Syn with WT Rab7A (final concentration for all 1.0×10^9 GC/ml). Cells were fixed (4% paraformaldehyde, 10 min, 20 °C) on DIV 15 for phospho- α Syn and LAMP1, and for PSD95 and MAP2 labeling. Cells were fixed on DIV 16 for detecting 53BP1. Cells were fixed or lysed on DIV 18 or DIV 20 for toxicity and comet assays.

2.3. Microfluidic chambers for axonal regeneration assay

The effect of WT and T22N Rab7 on α Syn-induced inhibition of axonal regeneration was measured as previously described (Park et al., 2006). Primary cortical neurons were seeded in silicone microfluidic devices with 450 μ m groove length according to the manufacturer's protocol. After bonding the microfluidic devices to a glass coverslip pre-coated with poly-L-ornithine, 100,000 cells (5.0×10^6 /ml) were plated in the cell compartment. The axonal side of the chamber was filled with 200 μ l complete Neurobasal medium. After attachment of the cells, 200 μ l medium was added in the cell compartment as well. One third of the medium was changed every third day on both sides. On DIV 12, neurons were infected with rAAV (final concentration for all 5×10^8 GC/ml). On DIV 15, axons were mechanically cut by an air bubble. Cells were fixed on DIV 20 and stained for tubulin (PRB-435P, followed by Alexa 555-labeled anti-mouse secondary antibody). Axonal length in the distal compartment was measured using ImageJ by drawing a segmented line along each individual axon projecting out of the microgroove.

2.4. Animals and surgery

All animal experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/

609/EEC) and approved by the Bioethical Committee of the KU Leuven (ECD project P067-2013 and P085-2014, Belgium). Eight-week-old female Wistar rats (Janvier, France, 200–250 g) were housed under a 12-h light and/or dark cycle with free access to pelleted food and tap water. All surgical procedures were performed using aseptic techniques. Rats were anaesthetized with ketamine (60 mg/kg, intraperitoneal (i.p.), Ketalar, Pfizer, Belgium) and medetomidine (0.4 mg/kg, i.p., Dormitor, Pfizer). 3 μ l of vector mix containing the rAAV A53T α Syn vector (9.0E11 GC/ml, injected genome copies 1,35E9) and rAAV HA-Rab7A T22N vector (9,3E11 GC/ml, injected genome copies 1,39E9) or the rAAV HA-Rab7A WT vector (9,3E11 GC/ml, injected genome copies 1,39E9) was injected in the substantia nigra (AP: -5.3 ; L: -2.0 ; DV: -7.2 from dura, using Bregma as reference) with a Hamilton syringe (Hamilton, Bonaduz, GR, Switzerland) as previously described (Van der Perren et al., 2015, 2016).

Forelimb use was quantified by the cylinder test four weeks after the unilateral rAAV-injection. Contacts made by each forepaw with the wall of a 20-cm-wide clear glass cylinder were scored from the videotapes by an observer blinded to the animal's identity. A total of 25 contacts were recorded for each animal, and the impaired (left) forelimb usage was expressed as a percentage of total forelimb contacts. Non-lesioned control rats score around 50% in this test.

Rats were sacrificed four weeks after the rAAV-injection with an overdose of sodium pentobarbital (200 mg/kg, i.p.) followed by intracardial perfusion with 4% paraformaldehyde in PBS. After post-fixation (4% paraformaldehyde, overnight), 50 μ m-thick coronal brain sections were cut with a vibrating microtome.

2.5. Immunohistochemical and immunofluorescent stainings

To quantify the number of dopaminergic neurons in the substantia nigra (SN) and the density of dopaminergic axon terminals (fibers) in the striatum, free-floating sections were stained for tyrosine hydroxylase (TH, Ab152). α Syn pathology was quantified after staining for α Syn phosphorylated at Serine 129 (phospho- α Syn, 11A5). Sections were pretreated with 3% hydrogen peroxide (10 min) and incubated with primary antibody in 10% normal goat serum overnight. Incubation with biotinylated anti-rabbit IgG (TH) and anti-mouse IgG (phospho- α Syn) secondary antibodies was followed by streptavidin - horseradish peroxidase complex. TH immunoreactivity was visualized using Vector SG as a chromogen and phospho- α Syn immunoreactivity was visualized using 3,3-diaminobenzidine (DAB).

To determine neuroinflammation, every sixth striatal section was stained for the astroglia marker glial fibrillary acidic protein (GFAP, ab4674) and for the microglia marker Iba1 (019–19,741) by incubation with primary antibodies at 4 °C overnight and fluorescently labeled secondary antibodies (Alexa 488 conjugated goat anti-chicken and Alexa 555 conjugated donkey anti-rabbit, 120 min at 20 °C). Sections were counterstained with Hoechst and mounted with Fluoromount-G.

To detect DNA damage, brain slices from the SN (minimum 3 sections per animal) were stained for 53BP1 (PA5–54565), human α Syn (ALX-804-258-L001) and the HA-tag of Rab7 (MMS-101P). After incubation with secondary antibodies (Alexa 488 conjugated anti-rat, Alexa 555 conjugated anti-rabbit, and Alexa 405 conjugated anti-mouse), sections were also mounted with Fluoromount-G.

Fixed primary neurons were permeabilized using 0.2% Triton X-100 in Tris-buffered saline (TBS, pH 7.4, 10 min). Non-specific sites were blocked by 2% bovine serum albumin in TBS (RT, 1 h). The following primary antibodies were applied in blocking solution (4 °C, overnight): Microtubule-associated protein 2 (MAP2, ab5392), Lysosomal-associated membrane protein 1 (LAMP1, ab208943), 53BP1 (as above), human α Syn (as above), phosphorylated α Syn (ab51253), PSD95 (Cell Signaling, 3450) and HA-tag (as above). Secondary antibodies were: Alexa 647 conjugated anti-chicken, Alexa 555 conjugated anti-rabbit, Alexa 488 conjugated anti-rat and Alexa 405 conjugate anti-mouse. Coverslips were mounted with Fluoromount G. Co-occurrence of

phospho- α Syn and LAMP1 signal was determined by using the Manders' overlap coefficient (ImageJ, JACoP plugin), as fraction of phospho- α Syn signal within LAMP1 signal as previously (Dunn et al., 2011; Szegő et al., 2019). Mean fluorescent intensity of phospho- α Syn signal was measured within the MAP2 positive area as previously (Szegő et al., 2021).

2.6. Analysis of dopaminergic neurons, α Syn pathology and gliosis

The number of cells in the SN positive for TH and phospho- α Syn was determined by stereological measurements using the Optical fractionator method in a computerized system as described before (Baekelandt et al., 2002) (Stereoinvestigator). Every fifth section throughout the entire SN was analyzed, 7 sections for each animal. The coefficient of error calculated according to the procedure of Schmitz and Hof (2005), varied between 0.05 and 0.10. The volume of striatal lesion was determined by quantifying the TH-positive terminals using the Cavalieri method (Stereologer). Every fifth section covering the entire extent of the striatum was included in the counting procedure, 7 sections for each animal. An investigator blinded to the different groups performed all the analyses.

For quantification of gliosis, fluorescent images were acquired from slices stained for GFAP and Iba1 using a 20 $\times$ objective (NA 0.8) with an Axio Imager 2 microscope (Zeiss) and using a 40 $\times$ objective (NA 0.95) with a Spinning Disk microscope (Zeiss). After thresholding individual images (separately for GFAP and Iba1 channels), the area fraction was determined by ImageJ from 3 to 5 sections per animal and from 10 images per section as described previously (Szegő et al., 2017).

For quantification of striatal phospho- α Syn pathology, images from 3-diaminobenzidine stained sections (minimum 4 sections per animal, and minimum 4 images per section) were acquired using a 20 $\times$ objective (NA 0.8) with an Axio Imager 2 microscope (Zeiss). 4 independent blinded investigators rated the pattern of phospho- α Syn signal in 40–60 randomly selected images per evaluator. Blinding was achieved by 5 digit codes. Images were rated with 0 when mostly longer segments were present, and with 1 when a punctate pattern or dotted lines dominated the image. The results were analyzed in a hierarchical nested design as previously (Szegő et al., 2012). The evaluator was set as a random factor in the generalized linear mixed model, and it had no effect on the result. From the same images, the amount of phospho- α Syn staining was quantified as area fraction of the DAB signal as described for GFAP and Iba1.

2.7. Triton X-100 solubility

For α Syn protein quantification and detection of Triton X-100 insoluble proteins, neurons were lysed in buffer containing 1% Triton X-100, 25 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA and protease inhibitors as previously described (Szegő et al., 2019). After centrifugation (14,000 g, 30 min, 4 °C), supernatant was used as Triton X-100 soluble fraction. The pellet (Triton X-100 insoluble fraction) was washed in ice-cold PBS, centrifuged again and re-dissolved with sonication (10 s) in 50 μ l buffer containing 2% SDS, 75 mM Tris, 15% glycerol, 3.75 mM EDTA pH 7.4 and protease inhibitors. 10 μ g of Triton X-100 soluble lysate or 10 μ l from the re-dissolved pellet were loaded onto a 4–20% Tris/glycine SDS gel for western blot analysis. After blocking, membranes were incubated first in the presence of antibodies against human α Syn (ALX-804-258-L001), HA tag (MMS-101P), PSD95 (3450) and alpha-tubulin (ab6046), then with horse radish peroxidase-conjugated secondary antibodies (donkey anti-rat, donkey anti-mouse and donkey anti-rabbit). Signal was visualized with chemiluminescent substrate and detected with Luminescent Image Analyzer.

2.8. Biochemical assays

To determine the toxic effect of α Syn in cultured neurons, the concentration of LDH was measured in the medium 24 h after the last

medium change using the Cytotoxicity Detection Kit (Roche) according to the manufacturer's protocol. Briefly, for each independent preparations, 3–4 technical replicates were used and averaged. Absorbances were measured at 492 nm, reference wavelength was 620 nm. Background (medium) absorbance was subtracted from all values, and values were normalized to control (non-treated) and to maximal lysed (treated with 2% Triton X-100, according to the protocol) cells. Caspase activity was measured with EnzChek Caspase-3 Assay Kit (Thermo Fisher). Apoptosis was induced with 100 μ M NMDA for 24 h. Cells were lysed and protein concentration determined (BCA assay). 100 μ M Z-DEVD-AMC substrate was added to a sample of 100 μ g protein. After 45 min incubation at 20 °C, fluorescence of cleaved Z-DEVD-AMC was acquired (Excitation/Emission: 342/441 nm; Infinite 200 Pro). MAP2 stained primary neurons were quantified as area fraction from 20 $\times$ images as described for GFAP and Iba1.

Mitochondrial membrane potential, cytoplasmic reactive oxygen species (ROS) production and mitochondrial ROS production were measured in separate assays as described previously (Szegő et al., 2019). Tetraethylbenzimidazolylcarbocyanine iodide (JC-1, 50 nM, 37 °C, 30 min) was used to measure the mitochondrial membrane potential. Fluorescence at 485/505 nm (excitation/emission) and 535/570 nm was detected by a plate reader (Infinite 200 Pro). After measuring the basal mitochondrial membrane potential, the mitochondrial uncoupler FCCP (1 mM) was used to induce a mitochondrial stress.

Cytoplasmic ROS was measured using dichlorodihydrofluorescein diacetate (DCFH-DA; 10 mM, 37C, 30 min), mitochondrial ROS was measured using MitoSOX (200 nM, 20 min, 37C). Fluorescence at 485/530 nm (DCFH-DA) or 510/580 nm (MitoSox) was measured with plate reader. To detect the response to stress-induced ROS production, preparations were treated with 1 mM FCCP (MitoSox) or with 1% H₂O₂ (DCFH-DA).

2.9. DNA damage

To determine DNA double strand breaks, the number of 53BP1-positive nuclear loci was counted in brain sections containing nigral neurons transduced with human α Syn (minimum 3 sections per animal, and minimum 3 images per section, 15–30 neurons per animal) or in primary neuronal cultures (3 repetitions, minimum 15 neurons per repetition /condition) as previously described (Milanese et al., 2018).

DNA single- and double-strand breaks were detected via single cell gel electrophoresis (comet) assay, according to the guidelines provided in (Oliveira et al., 2006). Adherent microscopy slides were coated with 1% low melting agarose (PeqLab). Primary neurons were harvested in PBS, mixed with 1% agarose in PBS and placed on the pre-coated slide. After agarose gelling, slides were submerged in lysis buffer (2.5 M NaCl, 100 mM Na₂EDTA, 0.1% Triton X-100, 10 mM TRIS, pH 10, 4 °C, 1 h) and denatured in alkaline electrophoresis buffer (0.3 M NaOH, 1 mM Na₂EDTA, pH > 13, 30 min). Electrophoresis was carried out for 25 min at 20 V in the same buffer. Slides were removed from the electrophoresis chamber, fixed in 70% ethanol and stained with 2.5 μ g/ml of propidium iodide for 20 min at 20 °C. Excess of stain was removed by rinsing the slides with distilled water. For each slide, at least 10 images were acquired using an epifluorescence microscope (Zeiss Axio Observer). For each comet, the “tail moment” was determined as the product of the percentage of DNA in the tail and the tail length using the Open Comet plugin for Image J as described previously (Paiva et al., 2017).

2.10. Statistical analysis

For in vitro experiments, “n” was set to the number of individual preparations. Within one experiment, mean of the technical replicates was determined for each treatment group. In animal experiments, “n” was set to the number of animals. No animals were excluded from the analysis. In graphs, markers represent individual preparations or individual animals, lines represent mean and standard deviation (SD) of all

individual preparations or all animals. Data normality was tested by the Shapiro-Wilk test and graphically by QQ plot (R). *t*-Test, one-way ANOVA or two-way ANOVA were performed using GraphPad Prism. Linear regression was performed using R (v 2.8.0). Binomial data from pattern rating was analyzed using generalized linear mixed-effects model fitted with binomial distribution. Evaluator, animal, section and image were used as random effects nested within each other (R package: lme4). *P* values are indicated in the graphs by symbols with * or # representing *p* < 0.05, ** or ## representing *p* < 0.01, *** or ### representing *p* < 0.001. Exact *p* values are given in the Figure legend.

3. Results

3.1. Rab7 decreases α Syn toxicity in primary neuronal cultures

α Syn toxicity was induced in primary neurons by expressing human A53T α Syn via rAAV transduction on day in vitro 12 (DIV 12). Transduction with the same amount of rAAV encoding GFP was used as a control. On DIV 20, cells were fixed and stained for the neuronal marker MAP2 (Fig. 1A). The area fraction covered by MAP2-positive neurons was quantified (Fig. 1B). Cultures transduced with rAAV- α Syn presented fewer neurons and a less dense network of neurites than control cultures transduced with rAAV-GFP. In addition, cultures transduced with rAAV- α Syn released more LDH into the culture medium than cultures transduced with rAAV-GFP (Fig. 1C), indicating plasma membrane damage and cell death. Neurons transduced with rAAV- α Syn were also more vulnerable than neurons transduced with rAAV-GFP: activation of caspase-3 after exposure to excitotoxic NMDA was much more pronounced in cultures transduced with rAAV- α Syn than in cultures transduced with rAAV-GFP (Supplemental Fig. S1A–C). Caspase 3 activation was measured biochemically and using immunocytochemistry.

Next we assessed whether Rab7 is able to reduce α Syn toxicity in primary neurons by co-transducing cultures with rAAVs encoding either WT Rab7, T22N Rab7 or GFP. The Rab7 mutant T22N binds Rab7 effectors but not guanosine-5'-triphosphate (GTP). GFP was used as a negative control for the two Rab7 variants to ensure an equal amount of vector transduction and protein synthesis. Transduction with rAAV-WT Rab7 alleviated the α Syn-induced reduction in MAP2-positive area by 20% (Fig. 1A, B). With T22N Rab7, the MAP2-positive area was not significantly different from the control condition and significantly lower than with WT Rab7 (Fig. 1A, B). Similarly, the release of LDH into the culture medium was significantly decreased by transduction with rAAV-WT Rab7 (Fig. 1C). With T22N Rab7, the LDH release was not significantly different from the control condition and significantly higher than with WT Rab7 (Fig. 1C). Furthermore, the NMDA-induced caspase-3 activation was lowered by WT Rab7 (Supplemental Fig. S1A). With T22N Rab7, the NMDA-induced caspase-3 activation was not significantly different from the control condition and significantly higher than with WT Rab7 (Supplemental Fig. S1A–C).

3.2. Rab7 decreases α Syn pathology in primary neuronal cultures

To characterize α Syn pathology in our neuronal culture model, we assayed the Triton X-100 solubility of human α Syn in neuronal cell lysates (Fig. 1D–G). In lysates prepared from cultures transduced with rAAV- α Syn, human α Syn was detected both in the Triton X-100 soluble fraction (Fig. 1D) and in the Triton X-100 insoluble fraction (Fig. 1E). In lysates prepared from cultures transduced with rAAV-GFP, human α Syn was not detected in relevant amounts (Fig. 1D, E). Co-expression of WT Rab7 (detected by the HA-tag in Fig. 1D) significantly reduced the amount of Triton X-100 insoluble α Syn (Fig. 1E,F). The amount of Triton X-100 soluble human α Syn was not decreased by WT Rab7 (Fig. 1D) but rather showed a slight, but not significant increase (quantified in Supplemental Fig. S2A). With T22N Rab7, Triton X-100 insoluble α Syn was not significantly different from the control condition and significantly

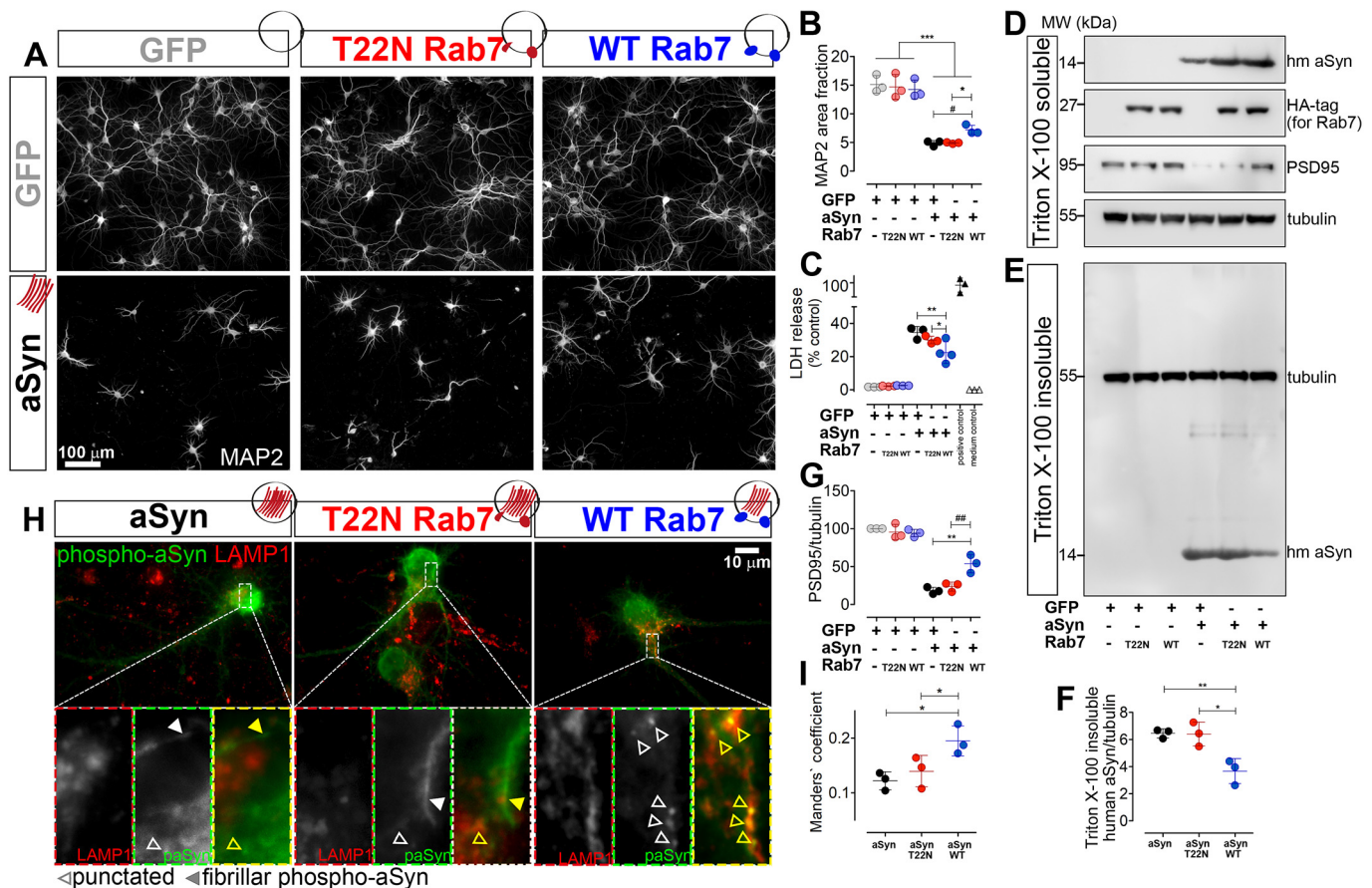


Fig. 1. WT Rab7 reduces α Syn toxicity in primary neurons.

(A) Representative images of primary neuronal cultures stained for the neuronal marker MAP2. Cultures were transduced by rAAV vectors as indicated. Scale bar: 100 μ m. (B) Area fraction positive for MAP2 in cultures treated as described in (A) (markers represent $n = 3$ preparations, mean $\pm$ SD; two-way ANOVA followed by Bonferroni post-hoc test: ***: $p = 0,00074$; *: $p = 0,0355$; #: $p = 0,0402$). (C) LDH release was measured in cultures treated as in (A) and normalized to the indicated positive and negative controls (markers represent $n = 3-4$ preparations, mean $\pm$ SD; two-way ANOVA followed by Bonferroni post-hoc test, **: $p = 0,00064$; *: $p = 0,0317$). Cells of the positive control were treated with 1% Triton X-100. Empty medium was used as negative control. (D) Representative immunoblot of the Triton X-100 soluble fraction of lysates obtained from neuronal cultures as in (A). Membranes were incubated with antibodies against human α Syn, HA-tag (for Rab7), the post-synaptic marker PSD95 and the neuronal loading control β III-tubulin. Quantification of human α Syn signal and uncropped membranes are in Supplemental Fig. S2). (E) Representative immunoblot of the Triton X-100 insoluble fraction of the neuronal lysates as in (D), run under denaturing conditions and incubated with antibodies against human α Syn and β III-tubulin. (F) Quantification of the human α Syn signal in the Triton X-100 insoluble fraction (markers represent $n = 3$ blots as in (E), mean $\pm$ SD, one-way ANOVA followed by Tukey's multiple comparison test, **: $p = 0,0063$; *: $p = 0,0271$). (G) Quantification of the PSD95 signal (normalized to β III-tubulin) from the Triton X-100 soluble fraction (markers represent $n = 3$ preparations as in (D), mean $\pm$ SD, two-way ANOVA followed by Bonferroni post-hoc test, **: $p = 0,0061$; #: $p = 0,0089$). PSD95 immunostaining is in Supplemental Fig. S3. (H) Representative images of neuronal cultures transduced with rAAV as depicted and stained for phospho- α Syn (green channel) and LAMP1 (red channel). Inserts show fibrillar (closed arrowheads) and punctated (open arrowheads) phospho- α Syn signals. Scale bar: 10 μ m. (I) Co-localization of phospho- α Syn and LAMP1 signals in the treated neuronal cultures expressed by the Manders' overlap coefficient (percentage of the total phospho- α Syn signal within the LAMP1 signal, $n = 3$ preparations, mean $\pm$ SD, one-way ANOVA, *: $p = 0,0184$ for α Syn vs. α Syn+WT Rab7; $p = 0,0365$ for α Syn+T22N Rab7 vs. α Syn+WT Rab7).

higher than with WT Rab7 (Fig. 1E, F); Triton X-100 soluble α Syn was similar to WT Rab7 (Fig. 1D, Supplemental Fig. S2A).

In order to corroborate the functional significance of α Syn aggregation on the neuronal network in culture, we quantified the amount of the postsynaptic protein PSD95 using immunoblotting (Fig. 1D,G), and the amount of PSD95 signal per neuron using immunocytochemistry (Supplemental Fig. S3). By both methods, transduction with rAAV- α Syn reduced the amount of PSD95 protein in neuronal cultures, and this effect was mitigated by WT Rab7 (Fig. 1D,G and Supplemental Fig. S3). In primary cultures transduced with rAAV-T22N Rab7, PSD95 was not significantly different from the control condition and significantly lower than with WT Rab7 (Fig. 1D, G and Supplemental Fig. S3).

Rab7 is known for orchestrating trafficking of autophagosomal and endosomal vesicles. Accordingly, we previously demonstrated in HEK293T cells that Rab7 promotes the processing of α Syn into acidic

vesicles such as autolysosomes (Dinter et al., 2016). To revisit this mode of Rab7 action, we determined in our primary neuronal cultures the fraction of phospho- α Syn present in LAMP1-positive vesicles. Neurons positive for phospho- α Syn were also positive for human α Syn (Supplemental Fig. S4), indicating that the pathology resulted from transduction with rAAV- α Syn. Most neurons positive for human α Syn showed fibrillary structures positive for phospho- α Syn (closed arrowheads in Fig. 1H). These structures are reminiscent of α Syn immunoreactivity observed after PFF-induced seeding of α Syn pathology in primary neurons (Szegő et al., 2021; Volpicelli-Daley et al., 2014). In addition, we observed phospho- α Syn-positive puncta that colocalized with LAMP1 (open arrowheads in Fig. 1H). Upon quantification, the fraction of phospho- α Syn signal that colocalized with LAMP1 signal was larger in cultures transduced with rAAV-WT Rab7 than in cultures transduced with rAA T22N Rab7 (Fig. 1I). This finding is consistent with the

hypothesis that Rab7 increases maturation of α Syn-containing autophagosomes and degradation of misfolded α Syn. In line with this hypothesis, rAAV-WT Rab7 transduced neurons contained less phosphorylated α Syn compared to rAAV-T22N Rab7 or rAAV-GFP transduced neurons (Supplemental Fig. S4C).

Taken together, these findings confirmed the protective effect of WT

Rab7 against α Syn pathology and the associated toxicity in mammalian neurons. The protective effects were not observed with T22N Rab7, indicating that GTP binding is necessary for this Rab7 effect. Without α Syn expression, WT Rab7 had no obvious effects on any of the measured parameters, confirming that its protective effect resulted from modulation of α Syn toxicity and not from direct, α Syn-independent

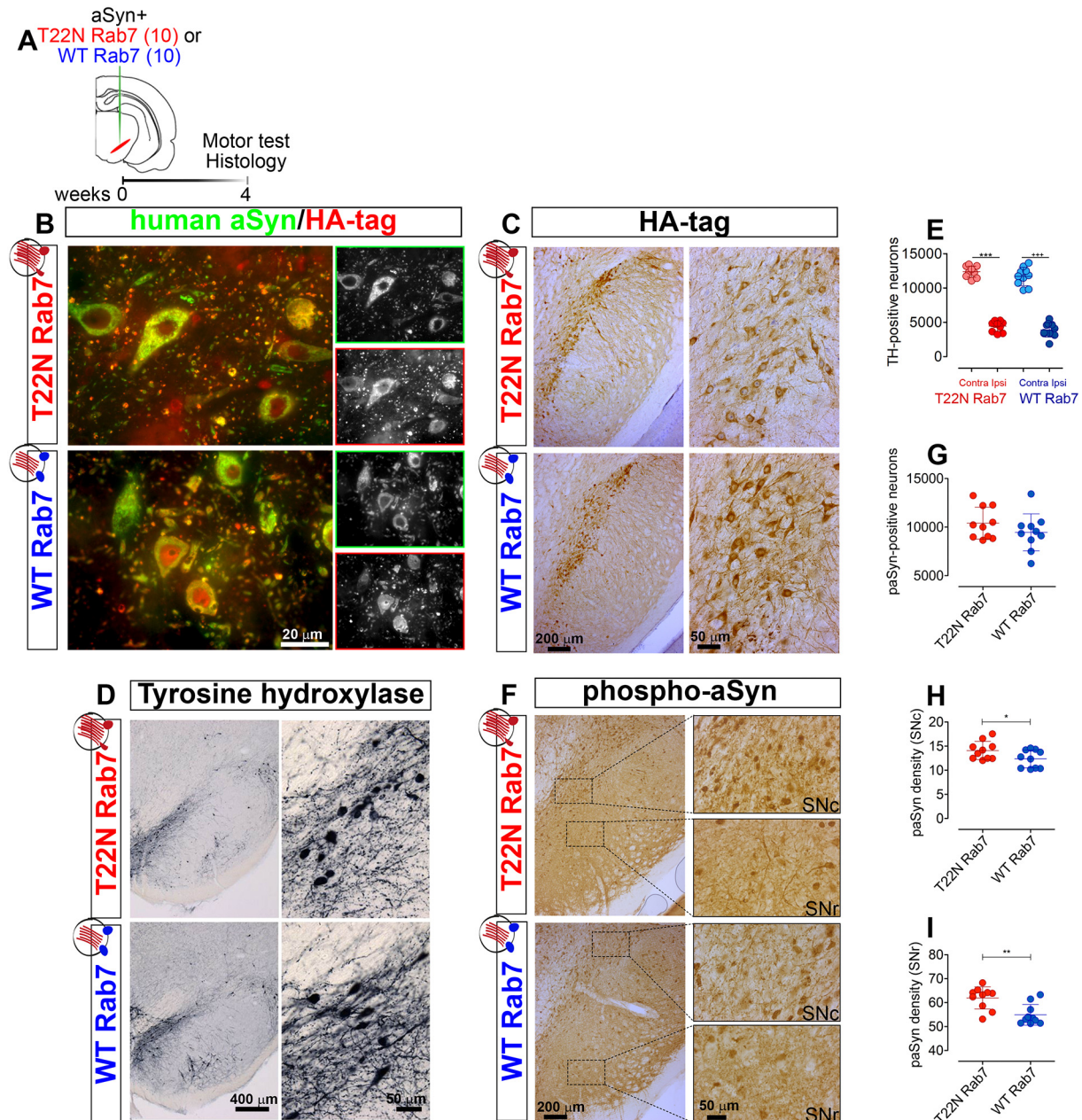


Fig. 2. WT Rab7 does not protect from loss of dopaminergic neuron somata, but reduces accumulation of phospho- α Syn in the neuropil.

(A) Experimental paradigm: rAAV encoding A53T α Syn and rAAV encoding either T22N Rab7 or WT Rab7 were injected into the right SN of rats (10 animals/group). Motor performance and α Syn-induced pathology were analyzed 4 weeks post injection. (B) Representative images of SN sections from animals injected with rAAV- α Syn and rAAV-T22N Rab7 or rAAV- α Syn and rAAV-WT Rab7 stained for human α Syn (green) and Rab7 (red, labeled through HA-tag), showing co-localization in neurons (yellow). Insets show the individual channels. Scale bar: 20 μ m. (C) Representative images of SN slices stained for Rab7 (through HA-tag) in the injected hemisphere. Scale bars: 200 μ m (left) and 50 μ m (right). Contralateral hemispheres are in Supplemental Fig. S5A. (D) Representative images of the injected SN stained for TH. Scale bars: 400 μ m (left) and 50 μ m (right). Contralateral hemispheres are in Supplemental Fig. S5B. (E) Stereological quantification of the number of TH-positive neurons in the SNc of the non-injected ("contra") and injected ("ipsi") hemispheres ($n = 10$, mean $\pm$ SD, two-way ANOVA; ***: $p = 0,00067$; +++: $p = 0,0085$). (F) Representative images of SN slices of the injected hemisphere stained for phospho- α Syn. Insets show the indicated areas within the SNc (top) and SNr (bottom). Scale bars: 200 μ m (left) and 50 μ m (right). (G) Stereological quantification of the number of phospho- α Syn-positive neurons in the injected SNc ($n = 10$, mean $\pm$ SD, t -test). (H) Quantification of phospho- α Syn-positive structures in the neuropil of the SNc ($n = 10$, mean $\pm$ SD, t -test, $p = 0,0498$). (I) Quantification of phospho- α Syn-positive structures in the SNr ($n = 10$, mean $\pm$ SD, t -test, $p = 0,0025$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

effects. Transduction with rAAV-T22N Rab7 was comparable to the transduction with rAAV-GFP. In particular, T22N Rab7 expression did not cause neuronal toxicity on its own or increase the extent of α Syn pathology. Thus, even though the T22N mutant is considered dominant negative (Spinosa et al., 2008), we did not observe dominant negative effects in our paradigms.

3.3. Rab7 does not protect dopaminergic neurons from α Syn toxicity, but it reduces the accumulation of phospho- α Syn in the substantia nigra

In order to further validate our findings, we tested the effect of WT Rab7 on α Syn-induced toxicity in dopaminergic neurons of rat SN. To do this, animals were injected with rAAV- α Syn combined with either rAAV-WT Rab7 or rAAV-T22N Rab7 (Fig. 2A) and sacrificed for analysis four weeks after the injection. Based on our results from primary neuronal

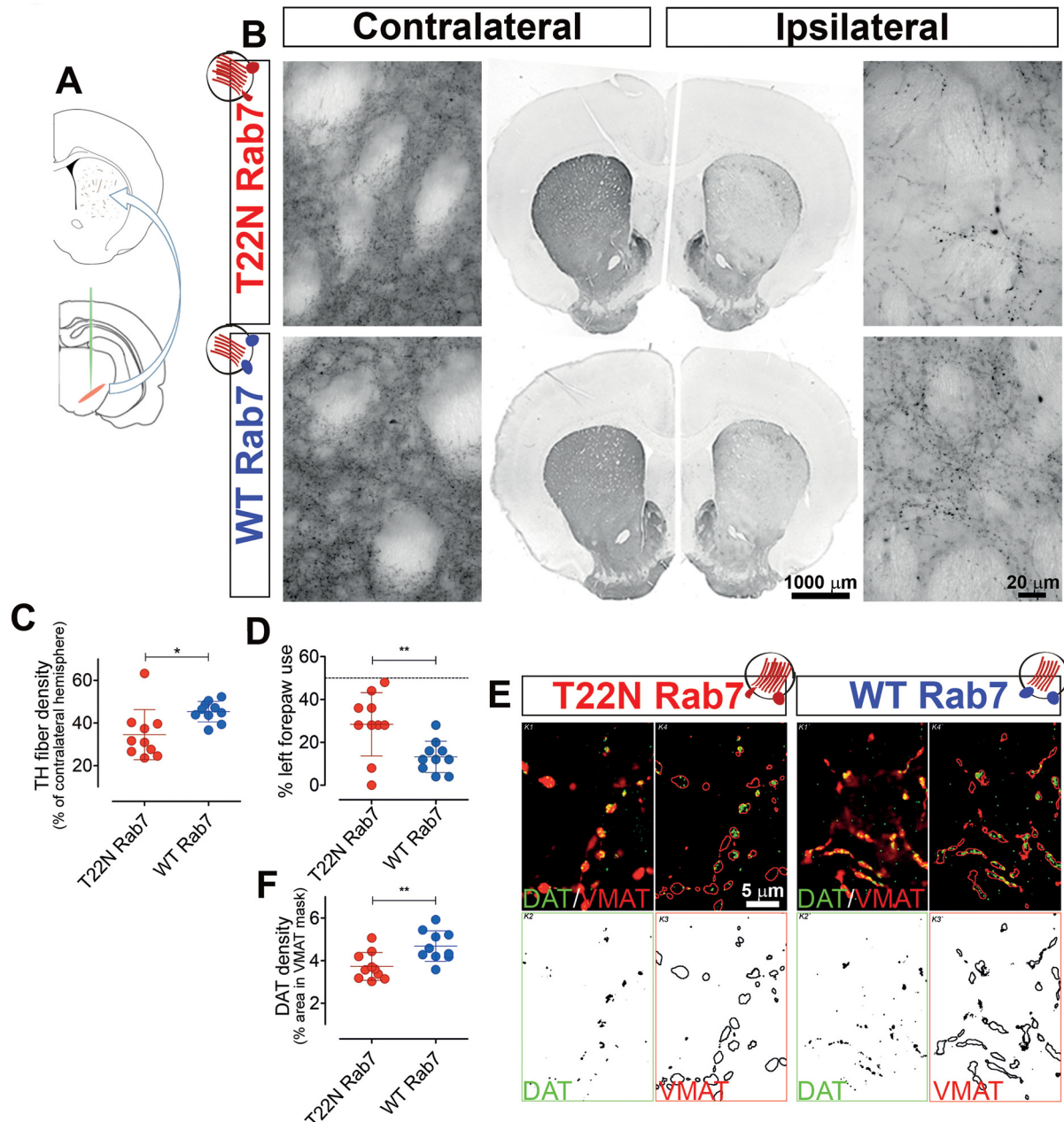


Fig. 3. WT Rab7 reduces α Syn-induced loss of dopaminergic axon terminals.

(A) Schematic presentation of the nigrostriatal pathway. Neurons in the substantia nigra were transduced by rAAV as described in Fig. 2. Dopaminergic neurons of the SNc extend their axon terminals to the striatum. (B) Representative images of striatal sections from animals injected with rAAV encoding α Syn and T22N Rab7 or α Syn and WT Rab7, stained for the dopaminergic marker tyrosine hydroxylase (TH). Scale bars: 1000 μ m (center) and 20 μ m (sides). (C) Quantification of the mean density of striatal dopaminergic axon terminals. Data are presented in relation to the contralateral hemisphere (n = 10, mean $\pm$ SD, t-test, p = 0,0149). (D) Quantification of the unilateral motor deficits 4 weeks after rAAV injection using the cylinder test (n = 10, mean $\pm$ SD, t-test, p = 0,0046). (E) Representative images of striatal sections stained for DAT (green pseudocolor) and VMAT2 (red pseudocolor) obtained 4 weeks after transduction with rAAV-T22N Rab7 (left) and rAAV-WT Rab7 (right) and in addition rAAV- α Syn. K1: Raw images (both channels merged). Scale bar: 5 μ m. K2: Binarized DAT signal. K3: Outline mask of the binarized image from the VMAT channel. K4: Merged image of K2 and K3. (F) Quantification of the DAT signal (from images as in K2) measured in regions defined by VMAT as in K3. (area fraction, n = 10, mean $\pm$ SD, t-test, p = 0,0058).

cultures, we chose T22N Rab7 as the negative control for WT Rab7 in order to have transduced DNA and protein as similar as possible.

As expected, we observed in the SN of the injected hemisphere neurons positive for both human α Syn (Fig. 2B, green) and the HA-tag of Rab7 (Fig. 2B, red); most neurons were positive for both. Four weeks after the injection, the number of HA-positive neurons in the SN was not

different between animals transduced with rAAV-WT Rab7 or rAAV-T22N Rab7 (Fig. 2C), and neurons with human α Syn or HA-tag expression were not observed in the contralateral hemisphere (Supplemental Fig. S5A).

Dopaminergic neurons were identified by staining for tyrosine hydroxylase (TH, Fig. 2D, Supplemental Fig. S5B). Stereological

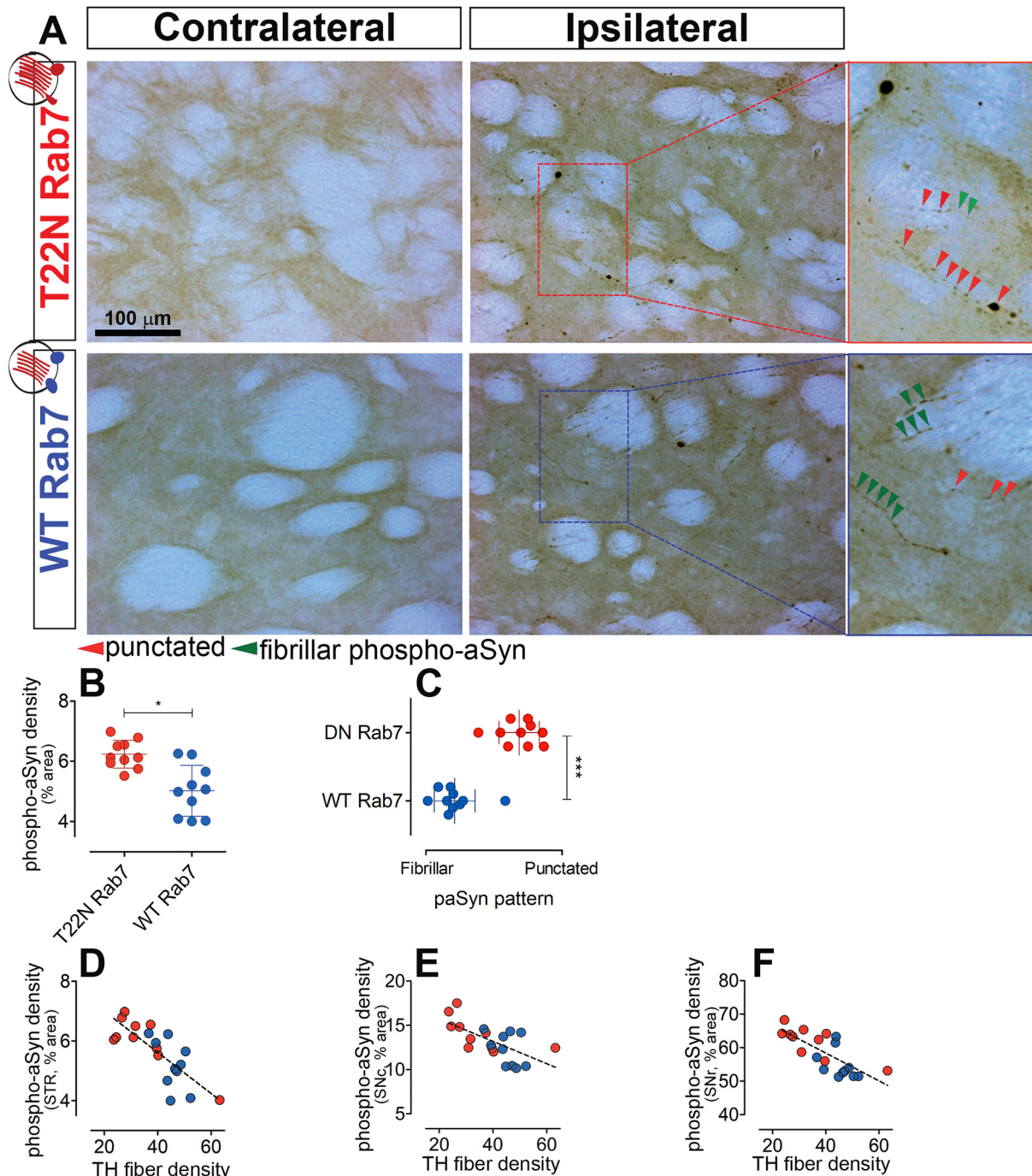


Fig. 4. WT Rab7 reduces α Syn pathology in the striatum.

(A) Representative images of striatal sections stained for phospho- α Syn. Typical fibrillar (green arrowheads) and pearl-like, punctated (red arrowheads) patterns are depicted in the inserts. Scale bar: 100 μ m. (B) Quantification of the density of phospho- α Syn-positive structures in the striatum (area fraction, $n = 10$, mean $\pm$ SD, t -test, $p = 0.0376$). (C) Quantification of the mean rating of the phospho- α Syn-positive structures as in (A). (Binomial rating: 0 = predominantly fibrillar, 1 = predominantly pearl-like pattern; $n = 10$, mean $\pm$ SD, logistic regression, $p = 0.00072$). (D) Linear regression between the density of dopaminergic axon terminals (from Fig. 3C) and the density of phospho- α Syn structures in the striatum (from B). ($p = 0.0005$, $r^2 = 0.6137$) (E) Linear regression of dopaminergic terminals in the striatum (from Fig. 3C) with the density of phospho- α Syn structures in the substantia nigra pars compacta (from Fig. 2H). ($p = 0.0022$, $r^2 = 0.4154$) (F) Linear regression of dopaminergic terminals in the striatum (from Fig. 3C) with the density of phospho- α Syn structures in the substantia nigra pars reticulata (from Fig. 2I). ($p = 0.0006$, $r^2 = 0.5773$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

quantification revealed a 65% loss of TH-positive nigral neurons upon transduction with rAAV- α Syn (Fig. 2D, E). In contrast to the protective effect of WT Rab7 in cultured neurons (Fig. 1), the numbers of dopaminergic neurons were not different between animals transduced with rAAV- α Syn + rAAV-T22N Rab7 and animals transduced with rAAV- α Syn + rAAV-WT Rab7 (Fig. 2E).

Next, we analyzed the effect of Rab7 on the number of neurons in the SN that are positive for phosphorylated α Syn (Fig. 2F), a hallmark of α Syn pathology (Anderson et al., 2006). Four weeks after rAAV injection, the number of phospho- α Syn positive cells in the SN was not different between animals transduced with rAAV- α Syn + rAAV-T22N Rab7 and animals transduced with rAAV- α Syn + rAAV-WT Rab7 (Fig. 2F, G). Yet, the density of phospho- α Syn-positive structures in the area between the neurons ("neuropil") quantified in the SN pars compacta (SNc, Fig. 2H), and in the SN pars reticulata (SNr, Fig. 2I) was significantly lower in animals transduced with rAAV-WT Rab7 than in animals transduced with rAAV-T22N Rab7. We assume that a significant part of the phospho- α Syn-positive structures in the SN neuropil correspond to dendrites of dopaminergic neurons (basal dendrites in the SNc and apical dendrites in the SNr), but axonal structures and non-dopaminergic neurons could contribute to the signal as well.

3.4. Rab7 protects striatal dopaminergic axon terminals from α Syn toxicity

Next, we characterized the effect of WT Rab7 and T22N Rab7 on the α Syn-induced loss of dopaminergic axon terminals ("fibers") in the striatum (Fig. 3A, B). In both groups, the density of striatal dopaminergic fibers was reduced to less than half of the density in the contralateral, non-injected hemisphere. Yet, the reduction was less pronounced in animals transduced with rAAV- α Syn + rAAV-WT Rab7 than in animals transduced with rAAV- α Syn + rAAV-T22N Rab7 (Fig. 3B, C). This 16% rescue is consistent with the protective effect of WT Rab7 on the density of the neuronal network in cultured neurons (Fig. 1B) and with the reduced density of phospho- α Syn in the SN neuropil (Fig. 2F). The extent of fiber loss in the striatum did not correlate with the loss of somata in the SN of the same animals (Supplemental Fig. S5C), but a differing response to toxic insults by somata of dopaminergic neurons in the SN and their axonal compartments in the striatum has been observed in numerous previous studies (Chung et al., 2009; Koch et al., 2015; Masliah et al., 2000).

Striatal α Syn pathology was observed only in the injected hemisphere (Fig. 4A). It was mostly detected along neurite-like structures (Fig. 4A, insets, green arrows). Double staining for phospho- α Syn and TH revealed an overlapping distribution (Supplemental Fig. S5D), confirming that the majority of phospho- α Syn in the striatum is localized in dopaminergic terminals. We observed both fibrillar phospho- α Syn staining patterns (Fig. 4A, green arrowheads) and punctuated phospho- α Syn staining patterns (red arrowheads) and assume that the punctuated pattern represents a more advanced stage of neurite degeneration. The overall density of phospho- α Syn-positive structures was significantly lower in animals transduced with rAAV-WT Rab7 compared to animals transduced with rAAV-T22N Rab7 (Fig. 4B). In addition, we determined qualitatively whether images showed predominantly a fibrillary or punctuated phospho- α Syn staining pattern. These blinded ratings revealed that the more advanced punctuated pattern was more prevalent in animals transduced with rAAV-T22N Rab7, while animals transduced with rAAV-WT Rab7 showed more of the fibrillary pattern (Fig. 4C). These findings are in line with the observed protective effect of WT Rab7 on the striatal dopaminergic axonal terminals (Fig. 3C), the reduced amount of Triton X-100 insoluble α Syn in cultured neurons (Fig. 1E), the differences in phospho- α Syn-positive dendrites in SNc and SNr (Figs. 2H, I), and with our previous finding that WT Rab7 can promote the degradation of α Syn aggregates (Dinter et al., 2016).

The significant negative correlation between the amount of striatal phospho- α Syn and the density of dopaminergic fibers (Fig. 4D) suggests

that α Syn pathology is closely linked to the degeneration of axon terminals. The strong correlation between the density of striatal dopaminergic fibers in each animal and the amount of phospho- α Syn in the SNc (Fig. 4E) and SNr (Fig. 4F) indicates that α Syn pathology affects the entire neuron.

Next, we assessed whether Rab7 affected motor performance. Forelimb use is regulated by the extracellular concentration of dopamine in the contralateral striatum. α Syn-induced motor deficits were therefore quantified by the cylinder test, carried out four weeks after rAAV injection. Unilateral transduction with rAAV- α Syn combined with either rAAV-WT Rab7 or rAAV-T22N Rab7 into the right hemisphere reduced left forepaw use in almost all animals (Fig. 3D), consistent with the fact that dopamine is secreted from nigrostriatal axon terminals and TH fibers were reduced in the right striatum (Fig. 3C). Surprisingly, the forelimb asymmetry was larger in animals transduced with rAAV-WT Rab7 than in animals transduced with rAAV-T22N Rab7 (Fig. 3D). This finding was unexpected given the blunted TH fiber loss with WT Rab7 (Fig. 3C). However, it could be explained by an effect of Rab7 on the dopamine transporter (DAT) (Eriksen et al., 2010; Wu et al., 2017). The DAT is responsible for dopamine reuptake and termination of dopamine signaling. Reduced DAT activity potentiates dopaminergic neurotransmission and can cause asymmetry in forelimb use (Lee et al., 2008). We co-stained striatal sections for DAT and vesicular monoamine transporter 2 (VMAT2) (Fig. 3E). The area of the DAT signal within VMAT2-positive fibers was significantly higher in animals transduced with rAAV-WT Rab7 than in animals transduced with rAAV-T22N Rab7 (Fig. 3F). This finding can potentially explain the observed asymmetry in forelimb use.

α Syn pathology induces the activation of glia cells, which can induce toxic as well as protective responses (Nichols et al., 2019; Pekny et al., 2014). Transduction with rAAV- α Syn induced microglia activation in the injected striatum, but there was no difference between animals transduced with WT Rab7 and T22N Rab7 (Supplemental Fig. S6A, B). For astroglia, the α Syn-induced activation was moderate in animals with T22N Rab7 and significantly higher in animals with WT Rab7 (Supplemental Fig. S6A, C). Yet, the lacking correlation between the astroglia reaction and dopaminergic fiber density (Supplemental Fig. S6D) argues against a direct role of astroglia.

3.5. Rab7 reduces α Syn-induced fiber loss by increasing viability of dopaminergic neurons

It is well established that dopaminergic axon terminals regenerate after toxic insults (Kowsky et al., 2007), and that α Syn can inhibit this regeneration (Szegő et al., 2012). In order to rule out that the differences in the density of striatal dopaminergic terminals in Fig. 3C results from differences in axon terminal regeneration, we investigated whether WT Rab7 affects axon regrowth. Primary neurons were seeded in microfluidic chambers that separate axons from somata. After rAAV transduction, axons were dissected and the length of regenerated axons was measured five days later. Neither T22N Rab7 nor WT Rab7 alone had an effect on regeneration (Supplemental Fig. S7). In contrast, α Syn significantly reduced axon regeneration. The length of regenerated axons was shorter in cultures transduced with WT Rab7 and α Syn than in cultures transduced with T22N Rab7 and α Syn. Differences in axon sprouting therefore do not account for the differences between the densities of dopaminergic terminals in rat striatum.

Accumulation of human α Syn impairs neuronal viability and neuronal functioning, which, in turn, causes further accumulation of toxic α Syn species. Therefore, mitigation of α Syn pathology by WT Rab7 overexpression should improve cellular functioning, increase viability and slow down neurite retraction. To test this hypothesis, we measured the amount of reactive oxygen species (ROS) and the mitochondrial membrane potential, two classical indicators of cellular functioning (Musgrove et al., 2019) in primary neurons transduced with rAAV- α Syn and rAAV-Rab7. ROS levels and the mitochondrial membrane potential

were normalized to control cultures transduced only by rAAV-GFP (represented as dashed lines in Figs. 5A–C). Cultures transduced with rAAV- α Syn showed about 20% more basal cytosolic ROS than cultures without α Syn (Fig. 5A), consistent with our earlier findings (Szegő et al., 2019). α Syn increased mitochondrial ROS to a similar extent (Fig. 5B), and reduced the mitochondrial membrane potential (Fig. 5C). In addition to these steady-state measurements, we tested neurons' response to an acute induction of oxidative and mitochondrial stress: Treatment with H_2O_2 increased cytoplasmic ROS beyond the extent observed with α Syn transduction alone (Fig. 5A). Similarly, treatment with the mitochondrial uncoupler FCCP increased mitochondrial ROS (Fig. 5B) and decreased the mitochondrial membrane potential (Fig. 5C).

The α Syn-induced increase in steady-state cytoplasmic ROS was not significantly affected by Rab7 (Fig. 5A), but handling stress-induced ROS was more efficient in cultures transduced with rAAV-WT Rab7 than in cultures transduced with rAAV-GFP or rAAV-T22N Rab7 (Fig. 5A). For mitochondrial ROS, both steady-state and stress-induced levels of mitochondrial ROS were lower in cultures transduced with rAAV-WT Rab7 than in cultures transduced with rAAV-GFP or rAAV-T22N Rab7 (Fig. 5B). Finally, transduction with rAAV-WT Rab7 stabilized the mitochondrial membrane potential as compared to rAAV-GFP and rAAV-T22N Rab7 - and mitigated the stress-induced reduction in the mitochondrial membrane potential (Fig. 5C). These findings suggest that the protective effects of WT Rab7 observed in cultured neurons and in the striatum could involve better handling of α Syn-induced oxidative damage and less mitochondrial impairment. In none of the measurements did T22N Rab7 differ from GFP (Fig. 5A–C), confirming that T22N Rab7 does not induce additional toxicity in this paradigm.

Aggregated α Syn and ROS induce DNA damage (Martin et al., 2006; Milanese et al., 2018; Paiva et al., 2017; Schaser et al., 2019). We therefore measured α Syn-induced DNA damage by the comet assay. Nuclei of neurons transduced with rAAV-GFP instead of rAAV- α Syn remained intact during electrophoresis (Fig. 5D), whereas a significant amount of fragmented DNA was mobilized into comet tails from nuclei of rAAV- α Syn transduced neurons (Fig. 5D). The α Syn-induced tail moment was significantly decreased by co-transduction with rAAV-WT Rab7 as compared to transduction with rAAV-GFP or rAAV-T22N Rab7 (Fig. 5D, E).

DNA breaks recruit specific repair proteins to the sites of the damage. Tumor suppressor p53-binding protein 1 (53BP1) is an effector of the canonical DNA damage response (Panier and Boulton, 2014), and 53BP1 foci can be used to quantify the extent of DNA damage. The number of 53BP1 foci was significantly higher in neurons transduced with rAAV- α Syn than in neurons transduced with the rAAV-GFP control (Fig. 5F, G). WT Rab7, but not T22N Rab7, reduced the number of 53BP1 foci (Fig. 5G), confirming the reduction of DNA fragmentation as reported by the comet assay (Fig. 5E). Both methods also confirmed that expression of T22N Rab7 per se did not induce DNA damage in neurons.

To confirm these Rab7 effects obtained in neuronal cultures, we determined the effect of α Syn and Rab7 on DNA damage in rat brain by quantifying the number of 53BP1 foci in neurons of rat SN (Fig. 6). Transduction with rAAV- α Syn increased the number of 53BP1 foci in SN neurons, and fewer 53BP1 foci were observed in animals transduced with rAAV-WT Rab7 than with rAAV-T22N Rab7 (Fig. 6A, B). The extent of DNA damage as reported by the number of 53BP foci correlated with the density of dopaminergic axon terminals in the striatum of the same animal (Fig. 6C), and similarly with the amount of phospho- α Syn in striatum (Fig. 6D), SNr (Fig. 6E) and SNc (Fig. 6F). These correlations confirm the close link between degeneration of axon terminals, aggregate load and DNA damage. In conclusion they suggest that WT Rab7 reduces DNA damage by reducing α Syn aggregates.

4. Discussion

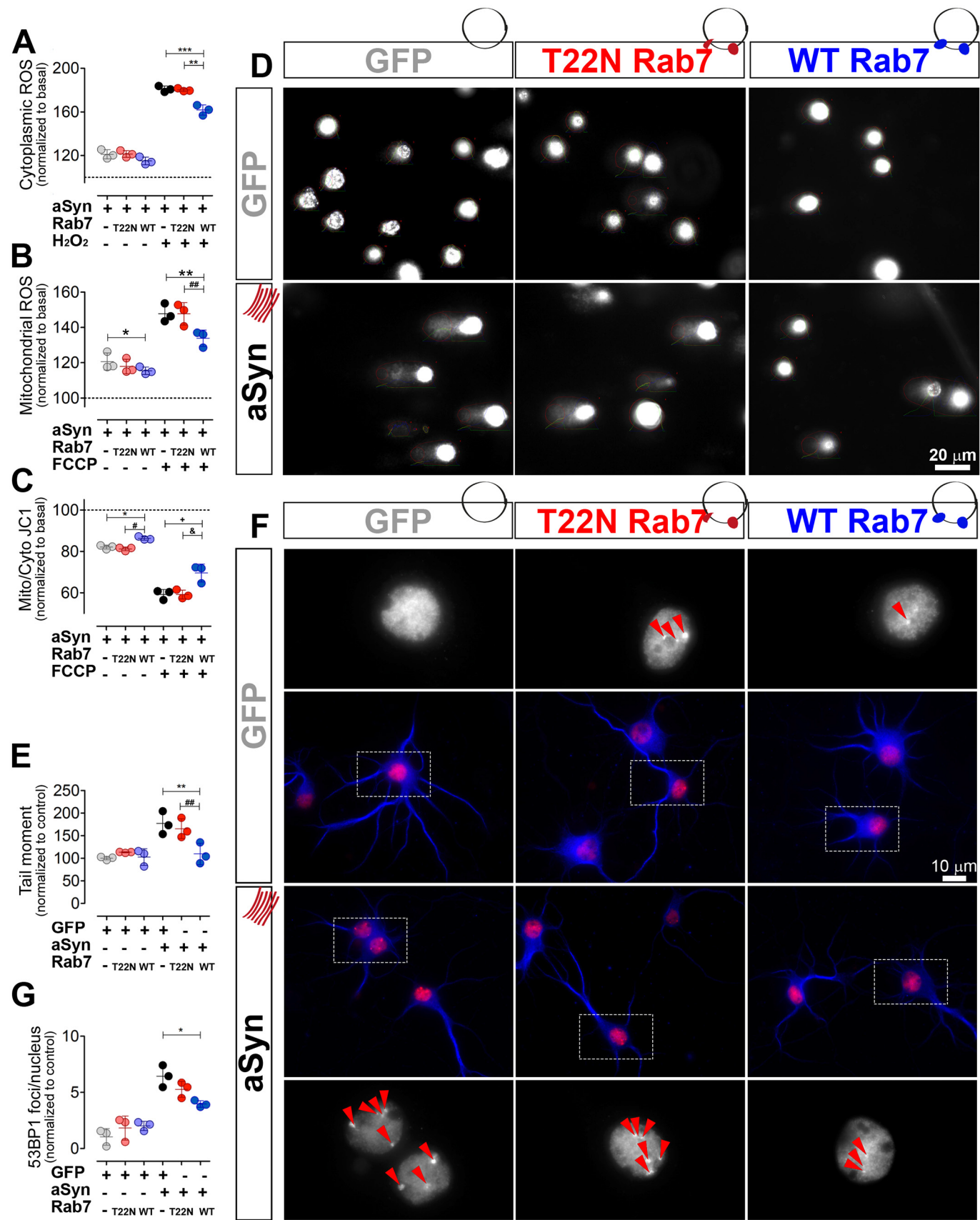
Here we report that the expression of WT Rab7 reduced α Syn pathology in primary neurons and in the rodent brain. Transduction with

rAAV-WT Rab7 abated α Syn-induced pathology. Primary cultures showed a higher density of MAP2-positive neurons and PSD95-positive synapses. They also showed decreased LDH release and caspase-3 activation. In animals, the lesion of dopaminergic axon terminals in the rat striatum and the extent of α Syn pathology were smaller. Pathological α Syn was decreased, and the fraction of α Syn in lysosomal vesicles increased by Rab7, indicating that toxic α Syn species are reduced via autolysosomal clearance. WT Rab7 reduced α Syn-induced ROS production, mitochondrial impairment and DNA damage, confirming the functional relevance of reducing α Syn pathology.

We induced α Syn pathology and toxicity by overexpressing A53T α Syn in the SN of rats and in primary mouse neurons using rAAV. Overexpression of α Syn in the rat brain induced a robust phenotype with a 60% reduction of both the number of dopaminergic somata in the SN (Fig. 2E) and dopaminergic axon terminals in the striatum (Fig. 3C) – in line with previous reports (Van der Perren et al., 2015). To promote the autolysosomal degradation of toxic α Syn species and to reduce α Syn dependent phenotypes, we expressed WT Rab7. The T22N mutant of Rab7 was used as a negative control. With WT Rab7, the densities of phospho- α Syn positive structures in SNc and SNr were reduced (Fig. 2), as was the density of phospho- α Syn positive axon terminals in the striatum (Fig. 4). In primary neurons, we observed a reduced amount of α Syn in the Triton X-100 insoluble fraction (Fig. 1E, F) and a higher colocalization between phospho- α Syn and LAMP1 with WT Rab7 compared to T22N Rab7 or GFP (Fig. 1H, I), indicating that Rab7 promotes the fusion of α Syn containing autophagosomes with lysosomes. This is consistent with our previous finding that Rab7 increases the fraction of α Syn in autolysosomes as reported by RFP-GFP tandem fluorescence (Dinter et al., 2016), and with the known promotion of autophagosome maturation by Rab7 (Gutierrez et al., 2004; Jäger et al., 2004; Wang et al., 2016a; Zhan et al., 2017; Zhao and Zhang, 2019).

T22N Rab7 did not increase the accumulation of insoluble α Syn (Fig. 1E, F) or induce toxicity (Fig. 1B, C, Fig. 5). This finding was unexpected given the reported dominant negative effects of the T22N mutation. It can be explained by assuming that Rab7 dependent aggregate clearance operates at low capacity without rAAV transduction (hence it cannot be reduced further by T22N Rab7). This hypothesis explains the lacking negative effect of T22N Rab7 and also the moderate but significant stimulation of aggregate clearance by transduction with rAAV-WT Rab7. A low baseline extent of Rab7-dependent aggregate clearance is consistent with the fact that there is no direct evidence that PD can be caused by a deficit in Rab7 activity except for the observation that mutations in the RAB7 gene are associated with melanoma (Alonso-Curbelo et al., 2014), which is more common in PD patients. Still, several other proteins involved in trafficking of endosomes and autophagosomes are linked to PD, including RabGEF1 and Arl8b (Bandres-Ciga et al., 2019). RabGEF1 activates Rab5 (Horiuchi et al., 1997) which characterizes early endosomes and is subsequently exchanged for Rab7. Both Rab7 and Arl8 are localized on late endosomes (Horiuchi et al., 1997; Jongsma et al., 2020; Marwaha et al., 2017), and Rab7 is exchanged for Arl8 during endosome maturation (Jongsma et al., 2020). Thus, the autophago-lysosomal pathway is clearly important during PD pathogenesis, and stimulating autophagosome maturation can be a viable therapeutic strategy even without a causative role of Rab7 during PD pathogenesis.

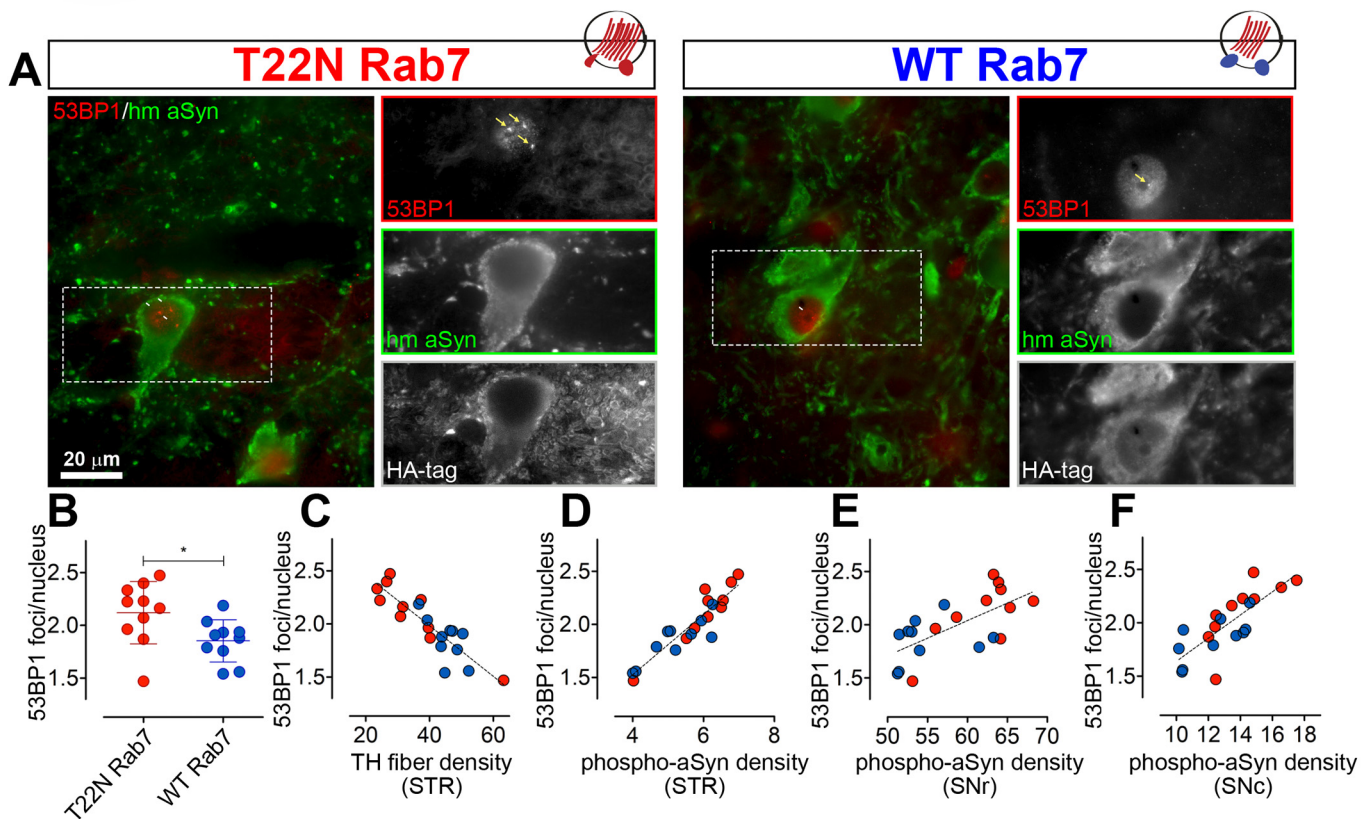
What is the mechanism of Rab7-induced aggregate clearance? Factors that mediate the Rab7 effects include the centrosomal protein ninein-like protein (Nlp), which enhances the interaction between Rab7 and FYCO1 (Xiao et al., 2021), PLEKHM1 (Marwaha et al., 2017) and Arl8 (Marwaha et al., 2017). Furthermore, we previously demonstrated that FYCO1 mediates the effect of Rab7 on the clearance of α Syn aggregates in HEK293T cells and in the fruit fly (Saridaki et al., 2018). Yet, the cellular mechanism underlying this effect has remained unclear. Rab7 primarily regulates autophagosome transport and fusion with lysosomes (Gutierrez et al., 2004; Jäger et al., 2004; Wang et al., 2016a; Zhao and Zhang, 2019). More recently, Rab7 has been implicated in the formation



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Fig. 5. WT Rab7 mitigates α Syn-induced oxidative stress and DNA damage in primary neurons.

(A–G) Primary neuronal cultures were transduced with rAAV encoding α Syn or GFP and in addition with rAAV encoding GFP, T22N Rab7 or WT Rab7 and analyzed four days (DNA repair site visualized by 53BP1, biochemical assays) or eight days (comet assay) later. (A) Cytoplasmic ROS determined using DCFH-DA and expressed relative to the mean of all control cultures (i.e. all cultures transduced with GFP instead of α Syn), represented by the dotted line. The “–” in the row for Rab7 stands for GFP. Additional oxidative stress was induced by adding 1% H_2O_2 where indicated ($n = 3$, mean $\pm$ SD, ANOVA followed by Bonferroni posthoc test; ***: $p = 0,00068$; **: $p = 0,0071$). (B) Mitochondrial ROS determined using MitoSOX and expressed relative to the mean of all control cultures (dotted line). Additional mitochondrial ROS were induced by adding 1 mM FCCP where indicated. ($n = 3$, mean $\pm$ SD, ANOVA followed by Bonferroni posthoc test; *: $p = 0,0408$; #: $p = 0,0058$; **: $p = 0,0069$). (C) Mitochondrial membrane potential measured by JC-1 expressed relative to the mean value of all control conditions (dotted line). FCCP was added where indicated ($n = 3$, mean $\pm$ SD, ANOVA followed by Bonferroni posthoc test; *: $p = 0,0433$; #: $p = 0,0395$; +: $p = 0,0296$; &: $p = 0,0247$). (D) Representative images of comet assays, i.e. neuronal nuclei embedded in agarose and stained by propidium iodide after electrophoresis. Scale bar: 20 μ m. (E) Mean tail moments determined for each preparation from minimal 10 images per preparation as in (D) ($n = 3$, mean $\pm$ SD, ANOVA followed by Bonferroni posthoc test; **: $p = 0,00317$; #: $p = 0,0053$). (F) Representative images of primary neurons stained for the DNA damage response protein 53BP1 (red) and MAP2 (blue) showing 53BP1-positive foci in the nuclei (red arrowheads) representing DNA damage sites. Scale bar: 10 μ m. Boxes depict the areas shown in the insets above and below the image. In the insets, only the 53BP1 channel is shown. (G) Mean number of 53BP1-foci per nucleus for each preparation obtained from minimal 15 neurons per condition ($n = 3$, mean $\pm$ SD, ANOVA followed by Bonferroni posthoc test; $p = 0,0109$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Fig. 6.** WT Rab7 mitigates α Syn-induced DNA damage in the substantia nigra.

(A) Representative images of substantia nigra slices stained for 53BP1 (red, top insert), human α Syn (green, middle insert) and Rab7 (HA tag, bottom insert), obtained from animals treated as described in Fig. 2A. Boxes are enlarged in the insets. DNA damage sites are depicted by yellow arrows. Scale bar 20 μ m. (B) Average number of 53BP1-foci per nucleus determined from images as in (A) and 15–30 neurons per animal ($n = 10$, mean $\pm$ SD, t -test, $p = 0,0148$). (C–F) Linear regression of the number of DNA damage sites (from B) with the density of dopaminergic axon terminals in the striatum (C, from Fig. 3C, $p = 0,0001$, $r^2 = 0,7725$), the density of phospho- α Syn structures in the striatum (D, from Fig. 4B, $p = 0,00011$, $r^2 = 0,8432$), the density of phospho- α Syn structures in the substantia nigra pars reticulata (E, from Fig. 2I, $p = 0,00015$, $r^2 = 0,4304$) and the density of phospho- α Syn structures in the pars compacta (F, from Fig. 2H, $p = 0,00014$, $r^2 = 0,6108$). Markers represent individual animals, $n = 10$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of membrane contact sites between the ER and autophagosomes (Elbaz-Alon et al., 2020) and in cholesterol transport (Meneses-Salas et al., 2020; van den Boomen et al., 2020). These processes could therefore contribute to Rab7-induced aggregate clearance.

Because Rab7 is a major signaling hub (Langemeyer et al., 2018; Wen et al., 2017; Zhang et al., 2009), directly increasing its activity might induce several off-target effects. One such off-target effect of Rab7 could explain our behavioral finding (Fig. 3J): Since Rab7 regulates endosomal trafficking, it could affect the abundance of DAT in striatal axon

terminals - as suggested by the findings in Fig. 3F. Specifically, higher DAT abundance in striatal axon terminals of animals transduced with WT Rab7 (Fig. 3F) could result in accelerated dopamine clearance and explain the reduced forelimb use (Fig. 3D). However, further experimental data is necessary to confirm this. In addition, the DAT is a secondary active transporter. Improved neuronal health in neurons transduced with WT Rab7 - as suggested by less DNA damage and better mitochondrial function - can also explain increased dopamine clearance and reduced forelimb use. Along similar lines, altered striatal dopamine

clearance underlies the paradoxically improved motor performance in a mouse model of α Syn pathology (Hunn et al., 2019). These observations highlight the Janus-faced consequences of dopaminergic terminal loss in the striatum: On the one hand, fewer terminals secrete less dopamine. On the other hand, reduced dopamine clearance and receptor sensitization potentiate dopamine effects. Accordingly, dyskinesias are induced more easily in dopamine-denervated striatum, and experimentally increasing striatal DAT activity can reduce dyskinesias (Tomas et al., 2016).

We observed a tight correlation between (1) the degeneration of dopaminergic terminals in the striatum, (2) α Syn pathology and (3) DNA damage (Fig. 4D, E and Fig. 6C, D). The fact that corresponding values for animals treated with either WT Rab7 or T22N Rab7 aligned on the same regression curve suggests that the protection of dopaminergic axon terminals by WT Rab7 was achieved by the reduction of α Syn pathology and the subsequent reduction of DNA damage. Cellular functioning and α Syn pathology are intricately linked, and the individual pathogenic steps potentiate each other. Our findings suggest that stimulating the rate limiting step of autolysosomal α Syn aggregate clearance by rAAV-mediated WT Rab7 expression breaks this vicious circle, and the improved cellular functioning limits further α Syn aggregation.

5. Conclusion

WT Rab7 reduced α Syn-induced degeneration of primary neurons and striatal dopaminergic axon terminals. WT Rab7 also lowered α Syn pathology and associated toxicity, including oxidative stress, mitochondrial impairment and DNA damage. These findings confirm that improving autophagosome maturation is a possible therapeutic strategy for PD.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.expneurol.2021.113900>.

Ethical approval and consent to participate

Animals were approved by local authorities (Bioethical Committee of the KU Leuven, Belgium, ECD project P067-2013 and P085-2014; Landesdirektion Sachsen, Germany, project 25-5131/476/15) and conducted in accordance with guidelines of the Federation for European Laboratory Animal Science Associations (FELASA).

Consent for publication

All authors approved the manuscript.

Availability of data and materials

The dataset supporting the conclusions of this article is included within the article and its additional files.

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Authors' contributions

AVDP, VB and BF conceived research. EMSz, CVDH, LH, AVDP performed research. EMSz, AVDP and BF wrote the manuscript, all authors contributed and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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