ORIGINAL ARTICLE

Heat Shock Protein 70 Reduces α -Synuclein-Induced Predegenerative Neuronal Dystrophy in the α -Synuclein Viral Gene Transfer Rat Model of Parkinson's Disease

Teresa C. Moloney,^{1,2,3} Rhona Hyland,^{1,2,3} Daniel O'Toole,² Alexia Paucard,^{1,2,3} Deniz Kirik,⁴ Aideen O'Doherty,² Adrienne M. Gorman^{2,3,5} & Eilís Dowd^{1,2,3}

¹ Department of Pharmacology & Therapeutics, National University of Ireland, Galway, Ireland

² National Centre for Biomedical Engineering Science (NCBES), National University of Ireland, Galway, Ireland

³ NCBES Galway Neuroscience Centre, National University of Ireland, Galway, Ireland

⁴ Department of Experimental Medical Science, Lund University, Lund, Sweden

⁵ Department of Biochemistry, National University of Ireland, Galway, Ireland

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Correspondence

E. Dowd, Department of Pharmacology & Therapeutics, National University of Ireland, Galway, Co. Galway, Ireland.

Tel.: +353 91 492776;

Fax: +353 91 525700;

E-mail: eilis.dowd@nuigalway.ie

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The first two authors contributed equally to this work.

SUMMARY

Aims: It has become increasingly evident that the nigrostriatal degeneration associated with Parkinson's disease initiates at the level of the axonal terminals in the putamen, and this nigrostriatal terminal dystrophy is either caused or exacerbated by the presence of α -synuclein immunopositive neuronal inclusions. Therefore, strategies aimed at reducing α -synuclein-induced early neuronal dystrophy may slow or halt the progression to overt nigrostriatal neurodegeneration. Thus, this study sought to determine if adeno-associated virus (AAV) mediated overexpression of two molecular chaperone heat shock proteins, namely Hsp27 or Hsp70, in the AAV- α -synuclein viral gene transfer rat model of Parkinson's disease could prevent α -synuclein-induced early neuronal pathology. **Methods:** Male Sprague–Dawley rats were intranigally coinjected with pathogenic (AAV- α -synuclein) and putative therapeutic (AAV-Hsp27 or AAV-Hsp70) viral vectors and were sacrificed 18 weeks postviral injection. **Results:** Intranigral injection of AAV- α -synuclein resulted in significant α -synuclein accumulation in the substantia nigra and striatal terminals which led to significant dystrophy of nigrostriatal dopaminergic neurons without overt nigrostriatal neurodegeneration. Coinjection of AAV-Hsp70, but not AAV-Hsp27, significantly reduced AAV- α -synuclein-induced neuronal dystrophy. **Conclusions:** These data confirm that overexpression of Hsp70 holds significant potential as a disease-modulating therapeutic approach for Parkinson's disease, with protective effects against early-onset α -synuclein-induced pathology demonstrated in the AAV- α -synuclein model.

Introduction

The severe and progressive loss of the nigrostriatal dopaminergic pathway is one of the distinguishing features of Parkinson's disease and this is thought to be largely driven by aberrant α -synuclein expression and aggregation [1–6]. In more recent years, evidence has accumulated to suggest that nigrostriatal neurodegeneration initiates in the axonal terminals in the striatum before progressing in a retrograde degenerative manner to the cell bodies in the substantia nigra [7–12]. This early-onset terminal pathology is characterized by the presence of morphologically aberrant tyrosine hydroxylase-immunoreactive axonal fibers (dystrophic neurites) in the putamen of early stage Parkinson's disease patients *postmortem*, which are associated with α -synuclein immunopositive neuronal inclusions [7,8]. In support of these clinical findings, several groups have reported that in viral α -synuclein models of Parkinson's disease [reviewed in 13], α -synuclein-induced axonal

swellings with truncated, dystrophic profiles appear in the tyrosine hydroxylase-immunopositive terminals in advance of overt dopaminergic cell loss [8,9,14]. Therefore, it is conceivable that strategies that can target this early α -synuclein-induced neuronal pathology may halt progression to overt nigrostriatal neurodegeneration.

One such approach is to harness the properties of heat shock proteins which, in their role as molecular chaperones, aid in the refolding of misfolded proteins [15,16]. Interestingly, Chu et al. [7] have reported a significant loss of heat shock protein (Hsp73; a member of the Hsp70 family) in the putamen of Parkinson's disease patients compared to age-matched controls, and this loss was most pronounced in neurons with α -synuclein inclusions. This suggests that a reduction of heat shock protein expression may actually contribute to the disease pathogenesis by reducing the diseased cells' ability to process α -synuclein.

Numerous *in vitro* and *in vivo* studies suggest that heat shock proteins, particularly Hsp27 and Hsp70, may be capable of

decreasing protein aggregation, including α -synuclein aggregation. *In vitro* aggregation assays demonstrate that Hsp27 and Hsp70 specifically bind and interfere with α -synuclein fibrillization kinetics inhibiting fibril assembly [17–20]. In primary neuronal cultures and neuroblastoma cell lines, Hsp27 and Hsp70 protect against α -synuclein-induced toxicity [21–24]. Notably, *in vivo* heat shock protein overexpression has potent protective effects in preclinical models of neurodegenerative diseases where protein aggregates, such as tauopathies and polyglutamine inclusions, are evident [25–30]. While a number of studies have investigated the effect of heat shock protein overexpression in various models of Parkinson's disease [31–38], to date there remains a paucity of preclinical information to judiciously evaluate the possible therapeutic potential that heat shock proteins may have on early α -synuclein-induced neuronal pathology.

Thus, the aim of this study was to determine if adeno-associated virus (AAV)-mediated overexpression of Hsp27 and Hsp70 could prevent AAV- α -synuclein-induced pathology, namely those changes associated with early-stage, predegenerative Parkinson's disease.

Methods

Animals

All procedures were carried out in accordance with European Union Directive 2010/63/EU and S.I. No. 543 of 2012 and were reviewed and approved by The Animal Care and Research Ethics Committee of the National University of Ireland, Galway. Male Sprague–Dawley rats, sourced from Charles Rivers, UK were used in all experiments.

Experimental Designs

Pilot Study

To establish if the viral transgenes employed were expressed in the appropriate neural cell type, that is, tyrosine hydroxylase immunopositive nigrostriatal cell bodies, eighteen male Sprague–Dawley rats (weighing 284 ± 3 g on the day of surgery) received intranigral injections of either control (AAV-GFP) or putative therapeutic (AAV-Hsp27 or AAV-Hsp70) viral vectors ($n = 6$ rats per group). Rats were sacrificed 2 weeks postviral injection by anesthetic overdose and transcardial perfusion, and brains were processed for immunohistochemical analyses. Overexpression of green fluorescent protein (GFP), Hsp27 or Hsp70 in tyrosine hydroxylase positive cell bodies was assessed by double immunofluorescence.

Main Study

A separate cohort of forty male Sprague–Dawley rats (weighing 284 ± 2 g on the day of surgery) were coinjected intranigral with pathogenic (AAV- α -synuclein) and putative therapeutic (AAV-Hsp27 or AAV-Hsp70) viral vectors to generate four final groups ($n = 10$ rats per group) as indicated in Table 1. Rats were sacrificed 18 weeks postviral injection by anesthetic overdose and transcardial perfusion, and brains were processed for immunohistochemical analyses. Eighteen weeks after virus infusion was cho-

Table 1 Final experimental groups used in the main experiment. Rats were given unilateral intranigral coinjection of two AAV vectors as indicated. All vectors were based on the AAV_{2/5} serotypes and the subscripts indicate the promoter used to drive transgene expression (SYN, human synapsin; CMV, cytomegalovirus). Viral titers were as follows: AAV-GFP_{SYN} and AAV- α -synuclein_{SYN}: 1.07×10^{10} drp/ μ L; AAV-GFP_{CMV}, AAV-Hsp27_{CMV} and AAV-Hsp70_{CMV}: 8.36×10^8 drp/ μ L. Coinjection of pathogenic and putative therapeutic viruses yielded a combined viral load of 1.15×10^{10} drp

Group	Pathogenic vector	Therapeutic vector	n
Intact	AAV-GFP _{SYN}	AAV-GFP _{CMV}	10
Lesion	AAV- α -synuclein _{SYN}	AAV-GFP _{CMV}	10
Hsp27	AAV- α -synuclein _{SYN}	AAV-Hsp27 _{CMV}	10
Hsp70	AAV- α -synuclein _{SYN}	AAV-Hsp70 _{CMV}	10

AAV, adeno-associated virus; GFP, green fluorescent protein.

sen as a predegenerative time-point for sacrifice because previous studies in our laboratory have revealed that AAV- α -synuclein-induced motor deficits begin to emerge strongly after this time [39]. The effect of Hsp27 and Hsp70 transgene expression on AAV- α -synuclein-induced α -synuclein expression, nigrostriatal dopaminergic integrity, and neuritic dystrophy was assessed by quantitative immunohistochemistry.

AAV Preparation

Adeno-associated virus vectors were prepared as described previously [39,40]. The genes for α -synuclein (normal human) and GFP were cloned into pTRUF flanked by AAV2 inverted terminal repeats under the human synapsin promoter. The human Hsp27 and Hsp70 genes were subcloned into the pAAV-MCS vector (Agilent Technologies Inc., Santa Clara, CA, USA), where the transgene is flanked by AAV2 inverted terminal repeats with constitutive expression under the cytomegalovirus promoter. These plasmids and the AAV5 helper plasmid, pDG-5, were purified for subsequent cell transfection. The AAV_{2/5} viral vectors were produced by cotransfecting HEK-293T cells with the relevant transgene plasmid and pDG-5, as described previously [41], for 48 h, by calcium phosphate precipitation. Viral vectors were purified by the treatment of the transfected cell pellet with a DNA endonuclease to degrade any non-encapsulated DNA, followed by a single step affinity chromatography purification of the freeze-thawed cell lysate, using Q-sepharose columns. Viral titers were established using real-time PCR and expressed as viral DNase-resistant particles (drp) per μ L (see Table 1 legend). Viruses were aliquoted into siliconized tubes and stored at -80°C .

Surgery

Unilateral intra-nigral surgery was conducted under isoflurane anesthesia (5% in O₂ for induction and 2% in O₂ for maintenance [42]) in a stereotaxic frame with the incisor bar set at -2.3 mm. The substantia nigra was targeted with viral infusions delivered at a rate of 1 μ L/min for 2 min (with an additional 2 min for diffusion) at stereotaxic coordinates A.P. -5.3 mm, M.L. $+2.0$ mm from bregma and D.V. -7.2 mm below dura.

Quantitative Immunohistochemistry

Rats were sacrificed by terminal anesthesia (50 mg/kg pentobarbital i.p.) and transcardially perfused with 100 mL heparinized saline followed by 150 mL of 4% paraformaldehyde. Brains were postfixed in 4% paraformaldehyde for 4 h and stored in a 25% sucrose plus 0.1% sodium azide solution. Serial coronal sections (40 μ m) were cut using a freezing sledge microtome and a 1:6 series was used for all quantitative immunohistochemistry. Because the viruses were injected unilaterally, the uninfected side served as a control for the immunohistochemical procedures. All quantitative immunohistochemistry was conducted by researchers blinded to the treatment of the rats.

Peroxidase-based immunohistochemical staining and immunofluorescent staining was completed as described previously [43,44]. In brief, following quenching of endogenous peroxidase activity where appropriate (using a solution of 3% hydrogen peroxide/10% methanol in distilled water) and blocking of non-specific secondary antibody binding (using 3% normal serum in Tris-buffered saline (TBS) with 0.2% Triton X-100 at room temperature for 1 h), sections were incubated overnight at room temperature with the appropriate primary antibody diluted in 1% normal serum in TBS with 0.2% Triton X-100 (mouse anti-tyrosine hydroxylase, MAB318, 1:1000; Millipore (Billerica, MA, USA); mouse anti- α -synuclein, AB36615, 1:1000; Abcam (Cambridge UK); sheep antityrosine hydroxylase, AB113, 1:500; Abcam; rabbit anti-GFP, A6455, 1:1000; Invitrogen (Carlsbad, CA, USA); mouse anti-Hsp27, SPA800, 1:1000; Stressgen (Victoria, BC, Canada); mouse anti-Hsp70, SPA8135, 1:1000; Stressgen; mouse anti- α -synuclein, AB36615, 1:1000; Abcam). Sections were then incubated for 3 h at room temperature with the appropriate fluorophore-labeled secondary antibody (donkey anti-sheep, A21098, 1:200; Invitrogen; goat anti-rabbit, A11034, 1:200; Invitrogen; goat anti-mouse, A11029, 1:200; Invitrogen) or, for peroxidase-based immunohistochemical staining, in a biotinylated secondary antibody for 3 h (horse anti-mouse, BA2001, 1:200; Vector, Burlingame, CA, USA), followed by a 2-h incubation in streptavidin-biotin-horseradish peroxidase solution (Vector). For α -synuclein immunofluorescent staining, horse anti-mouse (BA2001, 1:200; Vector) secondary antibody was used followed by a streptavidin-fluorophore conjugate (S11249, 1:1000; Invitrogen). For immunofluorescent staining, sections were then mounted onto gelatine-coated microscope slides and coverslipped using "Sigma Fluoromount" fluorescence mounting medium (Sigma-Aldrich, St. Louis, MO, USA). For peroxidase-based immunohistochemistry, sections were developed in 0.5% solution of diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) in Tris-buffer containing 0.3 μ L/mL of hydrogen peroxide. Sections were mounted on gelatin-coated microscope slides, dehydrated in ascending concentrations of alcohols, cleared in xylene and cover-slipped using DPX mountant (BDH chemicals).

Image Capture and Analysis

Photomicrographs of immunofluorescently stained sections were captured by an Olympus IX81 with a Hamamatsu C4742-80 digital camera. Triple immunopositive cell bodies (tyrosine hydroxylase,

GFP and α -synuclein) in the substantia nigra ipsilateral to the viral injection were quantified, and data were expressed as a percentage of the total number of tyrosine hydroxylase immunopositive cell bodies per section.

Photomicrographs of DAB-stained sections were captured under bright field illumination (using a Nikon DXM1200C digital camera mounted on a Nikon dissecting microscope) and all image analysis was completed using Image J software (ImageJ v1.41o; National Institute of Health, Bethesda, ML, USA). For quantification of AAV- α -synuclein-induced human α -synuclein expression, the number of α -synuclein immunopositive cell bodies in the substantia nigra and the optical density of α -synuclein immunopositive staining in the striatum were quantified, and data were expressed as a percentage of the intact side. For confirmation of the predegenerative status of this AAV- α -synuclein model, the impact of α -synuclein on the number of tyrosine hydroxylase immunopositive cell bodies in the substantia nigra and the optical density of tyrosine hydroxylase immunopositive staining in the striatum were quantified, and data were expressed as a percentage of the intact side. For quantification of AAV- α -synuclein-induced dystrophic neurites (identified as morphologically aberrant (swollen) tyrosine hydroxylase-immunoreactive axonal fibers), the number of tyrosine hydroxylase immunopositive dystrophic neurites in the injected striatum was quantified, and data were expressed as the number per field examined.

Statistical Analyses

Data were analyzed using a one-way ANOVA followed by *post hoc* Fisher's least significant difference test where appropriate. Differences between groups were considered statistically significant when $P < 0.05$. All data are expressed as mean $\pm$ SEM.

Results

Overexpression of Heat Shock Proteins within the Nigral Dopaminergic Neurons

In the initial pilot study, we sought to determine if the transgenes of interest, namely Hsp27 and Hsp70, were successfully overexpressed in the nigral dopaminergic neurons following a stereotaxic nigral injection of either AAV-Hsp27 or AAV-Hsp70 (Figure 1). Immunofluorescent staining confirmed that all transgenes of interest, that is, control GFP, Hsp27, and Hsp70, were strongly expressed following AAV delivery. Double immunofluorescent staining for the dopaminergic marker tyrosine hydroxylase revealed that all proteins were successfully colocalized within the dopaminergic neurons.

Coexpression of Transgene Protein Products within the Nigral Dopaminergic Neurons

In the main study, we first sought to determine if coinjection of the AAV vectors into the substantia nigra resulted in coexpression of the transgenes in the nigrostriatal dopaminergic neurons using triple immunofluorescence. Results confirmed that when AAV- α -synuclein and AAV-GFP were coinjected into the substantia nigra,

Figure 1 Overexpression of heat shock proteins within the nigral dopaminergic neurons. Representative fluorescent photomicrographs showing tyrosine hydroxylase immunopositive staining in the substantia nigra (left panels – red). Following a stereotaxic injection of AAV-GFP, AAV-Hsp27, or AAV-Hsp70 into the substantia nigra, the transgene protein products were strongly expressed 2 weeks postinjection (center left panels-green). Colocalization of GFP, Hsp27, and Hsp70 within the tyrosine hydroxylase positive cell bodies in the substantia nigra was confirmed in the merged images (center right and magnified right panels of boxed region). Scale bar left panel = 500 μ m, scale bar right panel = 125 μ m. AAV, adeno-associated virus; GFP, green fluorescent protein; TH, tyrosine hydroxylase.

Figure 2 Coexpression of transgene protein products within the nigral dopaminergic neurons. Following unilateral intranigral coinjection of two AAV vectors, triple immunofluorescent staining was used to determine if the different transgenes were coexpressed in the nigral dopaminergic neurons. **(A)** Representative fluorescent photomicrographs showing tyrosine hydroxylase (red), AAV-mediated GFP expression (green) and AAV-mediated human α -synuclein (blue) expression in the substantia nigra. Colocalization of α -synuclein and GFP within the tyrosine hydroxylase immunopositive cells in the substantia nigra is shown in the merged image. **(B)** Quantitative analysis of the triple immunopositive cells revealed that $32 \pm 2\%$ of nigral tyrosine hydroxylase immunopositive cell bodies were successfully transduced by both coinjected vectors. Scale bars: Top panels = 500 μ m; Bottom panels = 70 μ m. AAV, adeno-associated virus; GFP, green fluorescent protein; TH, tyrosine hydroxylase.

both transgenes were successfully coexpressed within nigral tyrosine hydroxylase immunopositive neurons (Figure 2A). Quantitative analysis of the triple immunopositive cells revealed that

$32 \pm 2\%$ of nigral tyrosine hydroxylase immunopositive cell bodies were successfully transduced by both coinjected viruses (Figure 2B).

AAV-Mediated Overexpression of Human α -Synuclein in Nigrostriatal Neurons

Quantitative immunohistochemical analysis for α -synuclein in the ventral midbrain and striatum was completed in order to confirm that the transgene was successfully delivered to the nigrostriatal neurons and expressed in both the cell bodies and terminals. In line with previous studies in our laboratory [39,40], injection of AAV- α -synuclein caused significant accumulation of the human α -synuclein protein in the ipsilateral nigrostriatal cell bodies (Figure 3Ai,Bi; Group, $F_{3,36} = 7.52$, $P < 0.01$) along with a significant increase in the density of human α -synuclein immunopositive staining in the striatal terminals (Figure 3Aii,Bii; Group, $F_{3,36} = 9.40$, $P < 0.001$). Although there was a tendency for reduction in α -synuclein levels at the terminal level, neither coexpression of Hsp27 nor Hsp70 had any significant impact on the overall levels of human α -synuclein expression in either the ventral mesencephalon (Figure 3Ai,Bi) or the striatal terminals (Figure 3Aii,Bii). It should also be noted that the α -synuclein antibody used also cross-reacted with endogenous rat α -synuclein

as α -synuclein immunoreactivity was also detected in the nigrostriatal cell bodies in the Intact group (Figure 3Ai,Bi).

Confirmation of the Predegenerative Status of this AAV- α -Synuclein Model

As indicated above, previous studies using the AAV- α -synuclein model in our laboratory have revealed that α -synuclein-induced motor dysfunction and neurodegeneration begins to emerge approximately 18 weeks after viral infusion [39]. Therefore, this time-point was chosen for sacrifice in the present study to ensure a presymptomatic, predegenerative AAV model. In line with this previous report, we did not observe any motor dysfunction in these animals up to this time-point (data not shown). To confirm the predegenerative status of this AAV- α -synuclein model, the impact of α -synuclein on tyrosine hydroxylase immunopositive cell bodies in the substantia nigra and terminals in the striatum was assessed. Injection of AAV- α -synuclein did not cause any loss of tyrosine hydroxylase immunopositive cell bodies from the substantia nigra (Figure 4Ai,Bi;

Figure 3 Adeno-associated virus (AAV)-mediated overexpression of human α -synuclein in nigrostriatal neurons. Immunohistochemical staining for α -synuclein revealed significant human α -synuclein accumulation in the substantia nigra (Ai) and in the striatal terminals (Aii) in the lesion group compared to those animals which received control viral injections. A similar pattern of human α -synuclein staining was also evident in animals that received both AAV- α -synuclein and AAV-Hsp27 or AAV-Hsp70 indicating that overall human α -synuclein expression was unaffected by coexpression of Hsp27 or Hsp70. Quantification of immunopositive staining revealed that injection of AAV- α -synuclein resulted in a significant increase in human α -synuclein expression in the nigral cell bodies (Bi) and in the striatal terminals (Bii). This level of human α -synuclein expression was not significantly affected by coexpression of Hsp27 or Hsp70. Scale bars: Left panels = 500 μ m; center panels = 100 μ m; right panels = 3 mm. ** $P < 0.01$ and *** $P < 0.001$ versus intact group by one-way ANOVA with post hoc Fisher's least significant difference test. All data are expressed as mean $\pm$ SEM AAV, adeno-associated virus; VM, ventral mesencephalon.

Figure 4 Confirmation of the predegenerative status of this AAV- α -synuclein model. Representative photomicrographs of immunohistochemical staining for tyrosine hydroxylase revealed that AAV- α -synuclein did not induce any loss of dopaminergic neurons from the substantia nigra (Ai) or striatal terminals (Aii). Coadministration of AAV-Hsp27 or AAV-Hsp70 with pathogenic AAV- α -synuclein also had no effect on nigrostriatal integrity. Quantification of tyrosine hydroxylase immunopositive cell bodies (Bi) and striatal terminals (Bii) confirmed that injection of AAV- α -synuclein alone or coinjection with putative therapeutic viruses AAV-Hsp27 or Hsp70 had no effect on nigrostriatal integrity. Scale bars: Left panels = 500 μ m; center panels = 100 μ m, right panels = 3 mm. All data are expressed as mean $\pm$ SEM. AAV, adeno-associated virus; SN, substantia nigra; TH, tyrosine hydroxylase.

Group, $F_{3,36} = 2.33$, $P = 0.09$, ns) or tyrosine hydroxylase immunopositive terminals from the striatum (Figure 4Aii,Bii; Group, $F_{3,36} = 1.45$, $P = 0.24$, ns). In the combined AAV- α -synuclein and AAV-Hsp27 or AAV-Hsp70 groups, there was no effect of coinjection of both viruses on nigrostriatal integrity at the level of the cell bodies (Figure 4Ai,Bi) or terminals (Figure 4Aii,Bii).

Overexpression of Hsp70 Reduces AAV- α -Synuclein-Induced Neuritic Dystrophy

In this predegenerative AAV model, tyrosine hydroxylase immunohistochemical staining revealed that injection of AAV- α -synuclein caused a significant increase in the numbers of dystrophic axon terminals present in the striatum (Figure 5A,B; Group, $F_{3,36} = 6.42$, $P < 0.01$). Quantification of the numbers of distorted axon terminals revealed that injection of AAV-Hsp27 did not significantly reduce the number of dystrophic neurites in the striatum. However, coinjection of AAV- α -synuclein and AAV-Hsp70 resulted in a statistically significant decrease in the number of dystrophic neurites present in the ipsilateral striatum (Figure 5A,B; *post hoc* Fisher's least significant difference confirmed Lesion $\approx$ Hsp27 > Hsp70 > Intact).

Discussion

The present experiment sought to determine if virally mediated heat shock protein overexpression could modulate α -synuclein-induced pathology in a predegenerative, early-stage model of Parkinson's disease, thereby providing a potential disease-modulating therapeutic approach for the human condition. To address this, we used the AAV human wild-type α -synuclein overexpression rat model of Parkinson's disease [14] and investigated the effect of AAV-mediated overexpression of Hsp27 and Hsp70 on α -synuclein-induced nigrostriatal pathology. This study found that viral overexpression of Hsp70, but not Hsp27, significantly reduced AAV- α -synuclein-induced axonal aberrations in the striatum. This finding supports the continued preclinical evaluation of heat shock protein based therapeutics to modulate α -synuclein-induced neuronal pathology.

For almost two decades, interest in α -synuclein as a potential disease-modulating therapeutic target in Parkinson's disease has increased steadily. This interest has led to the development of reliable animal models where genetic or viral overexpression of the wild-type or mutant protein can recapitulate many of the key features of the disease [reviewed in 13,45]. In this study, following an intra-nigral AAV- α -synuclein injection, significant overexpres-

Figure 5 Overexpression of Hsp70 reduces AAV- α -synuclein-induced neuritic dystrophy. **(A)** Representative photomicrographs of immunohistochemical staining for tyrosine hydroxylase revealed that AAV- α -synuclein caused nigrostriatal terminal pathology evidenced by morphologically aberrant, swollen axonal terminal in the striatum (arrows and inset). **(B)** Quantitative analysis confirmed that AAV- α -synuclein resulted in a significant increase in the expression of dystrophic neurites compared to intact controls. The levels of dystrophic neurites were unaffected by Hsp27 expression, however, Hsp70 expression resulted in a significant decrease in the levels of dystrophic neurites compared to the lesioned animals. Dystrophic neurites indicated by arrows. Scale bar = 50 μ m. * P < 0.05; ** P < 0.01 and *** P < 0.001 versus intact group, * P < 0.05 versus lesion group by one-way ANOVA with *post hoc* Fisher's least significant difference test. All data are expressed as mean $\pm$ SEM. AAV, adeno-associated virus.

sion of the human α -synuclein protein was evident both in the nigrostriatal cell bodies as well as the striatal terminals, thereby confirming successful transduction of the target neurons [46]. This increased α -synuclein expression induced formation of dystrophic neurites, a well-established characteristic of the AAV α -synucleinopathy model [9,14]. These dystrophic neurites are also present in the human condition, and are evident *postmortem* in the putamen of early stage Parkinson's disease patients before overt dopaminergic cell loss in the substantia nigra [8]. Moreover, a recent *postmortem* report of Parkinson's disease patients highlights that loss of dopaminergic markers of the axonal terminals in the dorsal putamen occurs rapidly and is virtually complete by 4 years postdiagnosis [47]. This indicates that there is a therapeutic "window-of-opportunity" for early neuroprotective intervention in Parkinson's disease, and it is becoming increasingly evident that the stage at which neuroprotective therapies are applied in Parkinson's disease patients will have significant influence on achieving satisfactory clinical outcome measures [48]. This emphasizes the importance of defining patients at the early disease phase, with premotor and prodromal Parkinson's disease, where enrollment in neuroprotective studies is more likely to preserve the nigrostriatal dopaminergic pathway. Given that we saw dystrophic neurites without overt dopaminergic cell loss in our preclinical model, this provided the possibility of evaluating putative therapeutic heat shock proteins in early-stage disease pathology when the nigrostriatal dopaminergic pathway is intact but dystrophic.

Given the expanse of *in vitro* and *in vivo* data supporting the potential of heat shock proteins to modulate misfolded proteins [49], we used viral vectors to overexpress Hsp27 and Hsp70 in this predegenerative AAV- α -synuclein animal model. Significant overexpression of the putative therapeutic proteins in the targeted dopaminergic cells was achieved using the AAV vectors. Overexpression of the putative therapeutic proteins reduced the appearance of dystrophic neurites in the striatum, with coinjection of AAV- α -synuclein and AAV-Hsp70, but not AAV-Hsp27, leading to a significant decrease in the number of dystrophic terminals in the striatum indicating that Hsp70 is protective against AAV- α -synuclein-induced pathology in the nigrostriatal terminals. Moreover,

because only 32% of tyrosine immunopositive nigral cells were cotransduced by the disease-causing and therapeutic viruses, it is possible that a greater therapeutic effect would have been observed if greater expression had been achieved.

The positive effects of Hsp70 overexpression on α -synuclein-induced pathology seen in our study are in line with previously reported studies on the protective effects of Hsp70 in models of Parkinson's disease. In a seminal study, Auluck et al. [31] demonstrated that directed coexpression of Hsp70 protected against dopaminergic neuronal loss associated with α -synuclein in *Drosophila melanogaster*. Similarly, it was demonstrated that cross breeding α -synuclein transgenic mice with Hsp70 transgenic mice led to a significant reduction in both the high molecular weight and detergent-insoluble α -synuclein species [33]. Given the numerous protective roles of Hsp70, it is difficult to definitively postulate as to the mechanism through which Hsp70 may act as a neuroprotectant in these α -synuclein overexpression animal models. One theory is based on a large body of literature highlighting that Hsp70 is capable of altering α -synuclein fibrillization kinetics, thus modulating the toxicity of α -synuclein. For example, Dedmon et al. [18] demonstrated that Hsp70 strongly inhibited α -synuclein fibril formation via preferential binding to prefibrillar species. Similarly, Huang et al. [19] demonstrated *in vitro* that Hsp70 is able to change the properties of fibril formation of α -synuclein by interactions with diverse intermediates of α -synuclein formed during fibrillization. Luk et al. [20] found that *in vitro* assembly of α -synuclein was efficiently inhibited by substoichiometric concentrations of purified Hsp70 in the absence of cofactors. In this same study, experiments using α -synuclein deletion mutants indicated that interactions between the Hsp70 substrate binding domain and the α -synuclein core hydrophobic region underlie assembly inhibition. Thus, there is substantial preclinical evidence that Hsp70 can reduce the neurotoxicity of α -synuclein by preventing the aggregation of this protein. However, it is important to note that it is not yet known if this molecular chaperone can assist with the clearance of pre-formed Lewy bodies which may be an important determinant of clinical efficacy in Parkinson's disease.

Particularly interesting, and highlighting a potential reason as to why we observed a beneficial effect on α -synuclein-induced pathology with Hsp70 but not Hsp27, is the ability of Hsp70 to chaperone α -synuclein extracellularly. Danzer et al. [21] reported that α -synuclein is present in its oligomeric form in extracellular space, from where it taken up by neighboring cells and mediates its toxic effects (contributing to theory of prion mediated transfer of α -synuclein disease pathology [50,51]). In their study, Hsp70 chaperoned α -synuclein in the extracellular space and prevented extracellular oligomer formation. The authors show that Hsp70's rescue of oligomer-induced toxicity is accompanied by a concomitant modification of the α -synuclein oligomeric species in the extracellular space. Thus, it is intriguing to speculate that this extracellular chaperone role of Hsp70 [52], a phenomenon not yet reported for Hsp27, could have been partly responsible for the beneficial effects seen in this study.

In toxin models of Parkinson's disease, Hsp70 has also shown to have positive effects on pathology. In an MPTP Parkinson's disease model, AAV-Hsp70 gene transfer significantly protected the mouse dopaminergic system against MPTP-induced dopamine neuron loss and the associated decline in striatal dopamine levels and tyrosine hydroxylase-positive fibers [32]. Systemic application of cell-permeable Hsp70 has also been shown to be protective for dopamine neurons of the substantia nigra against subacute toxicity of MPTP [37]. The mechanisms through which Hsp70 appears to be neuroprotectant in these models may reflect Hsp70s ability to regulate the apoptotic pathway. Hsp70 is capable of binding the caspase-recruitment domain of Apaf-1 thereby preventing recruitment of procaspase-9 to the apoptosome complex and blocking the assembly of a functional apoptosome [53,54]. Hsp70 can modulate apoptosis inducing factor activity [55–57], interfere with the Bid-dependent apoptotic pathway via inhibition of JNK [58] and inhibit the death-associated permeabilization of lysosomes [59]. Taken together, a number of *in vivo* studies indicate the significant potential of Hsp70 as a potential disease modifying, neuroprotective target for Parkinson's disease, while its precise mechanism of action in these models remains to be fully elucidated.

In this study, Hsp27 overexpression did not reduce dystrophic neurites in the striatum compared to the AAV- α -synuclein lesion

group. To our knowledge, no *in vivo* studies have been completed determining the effect of Hsp27 on α -synuclein aggregation making it difficult to compare our results in an *in vivo* context. Overexpression of Hsp27 in a chronic model of Huntington's disease, the R2/6 transgenic mouse, where intraneuronal polyglutamine protein aggregations are characteristic, did not prevent the formation of aggregates or the progression of the disease [60]. However, in other *in vitro* and *in vivo* models of Huntington's disease, overexpression of Hsp27 has been shown to decrease cell death without reducing the formation of aggregations [61,62]. In our experimental paradigm, it is conceivable that the levels of expression of Hsp27 that we achieved were not sufficient to have an appreciable protective effect. Thus, while a number of studies suggest that Hsp27 has protective effects against α -synuclein *in vitro* [17,22], our limited *in vivo* effect suggests that continued preclinical studies are warranted to establish its potential.

Conclusion

As dystrophic terminals with degenerating bulbs are thought to precede neuronal loss in Parkinson's disease [9,63], efforts to delay axonal dysfunction may preserve nigrostriatal connectivity thereby allowing afflicted patients to maintain an improved quality of life. In this experiment, Hsp70 significantly reduced the number of dystrophic neurites in the striatum in an early-stage, predegenerative model of Parkinson's disease. These data suggest that overexpression of Hsp70 holds significant potential as a disease-modulating therapeutic approach for Parkinson's disease.

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Conflict of Interest

The authors declare no conflict of interest.

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