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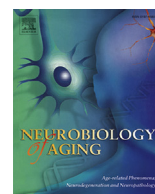

The loss of glucose-regulated protein 78 (GRP78) during normal aging or from siRNA knockdown augments human alpha-synuclein (α-syn) toxicity to rat nigral neurons

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ABSTRACT

Age-related structural changes and gradual loss of key enzymes significantly affect the ability of the endoplasmic reticulum (ER) to facilitate proper protein folding and maintain homeostasis. In this work, we present several lines of evidence supporting the hypothesis that the age-related decline in expression of the ER chaperone glucose-regulated protein 78 (GRP78) could be related to the development of Parkinson's disease. We first determined that old (24 months) rats exhibit significantly lower levels of GRP78 protein in the nigrostriatal system as compared with young (2 months) animals. Then using recombinant adeno-associate virus-mediated gene transfer, we found that GRP78 downregulation by specific small interfering RNAs (siRNAs) aggravates alpha-synuclein (α-syn) neurotoxicity in nigral dopamine (DA) neurons. Moreover, the degree of chaperone decline corresponds with the severity of neurodegeneration. Additionally, comparative analysis of nigral tissues obtained from old and young rats revealed that aging affects the capacity of nigral DA cells to upregulate endogenous GRP78 protein in response to human α-syn neurotoxicity. Finally, we demonstrated that a sustained increase of GRP78 protein over the course of 9 months protected aging nigral DA neurons in the α-syn-induced rat model of Parkinson's-like neurodegeneration. Our data indicate that the ER chaperone GRP78 may have therapeutic potential for preventing and/or slowing age-related neurodegeneration.
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1. Introduction

Activation of the endoplasmic reticulum (ER) stress response, also known as the unfolded protein response (UPR), and accumulation of intracellular or extracellular protein aggregates are common to many age-related neurodegenerative disorders, including Parkinson's disease (PD) (Hetz, 2012; Hoozemans et al., 2007, 2012; Muchowski and Wacker, 2005; Naidoo and Brown, 2012). Glucose-regulated protein 78 (GRP78), also known as BiP, is a key mediator of the UPR. It is a detector and regulator of the ER stress response that dissociates from 3 main "stress mediator" proteins when confronted with unfolded/misfolded proteins, thus activating them and the UPR. These stress sensors include activating transcription factor 6 (ATF6), inositol requiring protein 1, and Protein Kinase RNA-like endoplasmic reticulum kinase (PERK). Early, pro-survival UPR signaling attempts to restore ER homeostasis. If this fails, the subsequent proapoptotic (late) UPR signaling tries to kill the malfunctioning cell, and involves crosstalk between the ER and mitochondria (Rutkowski and Kaufman, 2004; Szegezdi et al., 2009).

A number of age-related changes in cellular structure and function can predispose individuals to PD. Among these is a sustained increase in alpha-synuclein (α-syn) protein, which when

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combined with the less efficient proteasome system in aging individuals leads to accumulation of misfolded protein (Auluck et al., 2010). This age-related accumulation of α-syn and an accompanying loss of tyrosine hydroxylase (TH)-positive (TH+) neurons were both previously documented in monkeys and humans (Chu and Kordower, 2007; Kahle et al., 2000). Overexpression of α-syn by a recombinant adeno-associate virus (rAAV) vector in the substantia nigra pars compacta (SNc) has also been shown to trigger a progressive nigrostriatal degeneration, which featured α-syn-positive cytoplasmic and axonal inclusions. This degeneration resulted in dystrophic and fragmented neuritis and ultimately led to cell death in both rat and mouse models of PD (Kahle et al., 2000; Kirik et al., 2002; Masliah et al., 2000). Although it appears that α-syn protein is less efficiently cleared from the cytosol, leading to its accumulation and Lewy body formation, the exact mechanism by which α-syn triggers neurodegeneration remains unclear.

Immunoreactivity for markers of UPR activation, such as phosphorylated PERK and eIF2α, was previously found in post-mortem tissues of PD patients but not control cases (Hoozemans et al., 2007), indicating UPR involvement that was unique to the diseased state. The accumulation of phosphorylated PERK also colocalized with accumulations of α-syn (Hoozemans et al., 2007), which was not surprising given that ER accumulation of α-syn was shown to trigger the UPR and subsequent proapoptotic changes (Colla et al., 2012a, 2012b). In addition to describing a close association between UPR signaling and α-syn accumulation and aggregation, there have also been reports demonstrating the potential for attenuating protein misfolding through pharmacological manipulation of ER stress in animal models of PD (Colla et al., 2012b; Matus et al., 2008). Despite these findings, little remains known about the age-related changes in individual UPR pathways and stress markers, which are associated with PD.

The age-related loss of critical UPR components and structural changes in the ER significantly affect its ability to control proper protein folding and maintain homeostasis (Naidoo and Brown, 2012). This breakdown in ER function is believed to occur, in part, from the oxidative wear of key ER resident proteins such as GRP78, PDI, calnexin, and GRP94 during aging (Hinds and McNelly, 1978; Nuss et al., 2008).

Old mice (20–24 months) have demonstrated a 20% decrease in GRP78 adenosine triphosphatase activity, as compared with young (3–5 months) mice, which is consistent with a 2-fold increase in GRP78 carbonylation. Such findings support the hypothesis that loss of ER or other cellular functions, often seen in age-related diseases, is caused by the life-long accumulation of oxidative damage to key proteins (Nuss et al., 2008). Another study by Erickson et al. (2006) describes a decline of ≤73% in GRP78 messenger RNA (mRNA) in old (900 days old) versus young (21 days old) rats, suggesting the loss of GRP78 activity, and the associated physiological declines occur at both the protein and transcript levels (Erickson et al., 2006). This suggests that the loss of GRP78 function could be a predisposing factor for neurodegenerative disorders associated with age (Naidoo and Brown, 2012).

We have previously demonstrated (Gorbatyuk et al., 2012) the involvement of UPR signaling in the degeneration of nigral dopamine (DA) cells caused by α-syn-induced pathology in rats. Parkinson-like neurodegeneration was induced by rAAV-mediated expression of human α-syn in the rat SNc. We found that the mechanism of α-syn neurotoxicity in nigral DA neurons was associated with an activation of the UPR. This increase in α-syn correlated with an increase in markers for 2 of the 3 pathways in the UPR at 4 weeks after injection, when α-syn expression reached maximum levels but significant neurodegeneration had not yet occurred. We observed a 23% elevation in phosphorylated eIf2α protein levels, a 9-fold induction in ATF4 (PERK pathway), and a greater than 2-fold elevation of the cleaved form of pATF6 protein (ATF6 pathway). These increases were also accompanied by a 3-fold elevation of proapoptotic CCAAT-enhancer-binding protein homologous protein (CHOP) protein that is known to be a downstream marker of the PERK and ATF6 pathways. Elevation of this protein is known to directly promote apoptosis and is thus perhaps responsible for DA cell death. Thus, we have concluded that overexpression of human α-syn activates the UPR signaling in the SNc.

In the present study, we attempted to answer the question: does a decline in GRP78 during aging make nigral neurons more susceptible to human α-syn cytotoxicity? To this end, we used rAAV-mediated transduction to simultaneously express human α-syn and anti-GRP78 specific small interfering RNAs (siRNAs) in the rat SNc. Because aging sees the decline of numerous genes that could contribute to nigral degeneration, we conducted our experiments in young (2 months) animals so as to isolate the effects of GRP78 reduction from that of other genes. We also tested the hypothesis that a long-term increase in GRP78 expression could protect nigral DA neurons against α-syn cytotoxicity in older animals.

2. Materials and methods

2.1. rAAV vectors

All vectors have been packaged in AAV5 capsid and purified as described previously (Zolotukhin et al., 2002). DNA sequences (Supplementary Table 1) encoding small hairpin RNA (shRNA) targeting GRP78 under the control of human U6 promoter were excised with EcoRI/SalI from the respective plasmid DNAs pGFP-V-RS supplied by the provider (OriGene). The EcoRI site was converted into SalI by adding the respective adapter sequence. The fragment was then subcloned into the SalI site of pTR-UF11 in the clockwise orientation, downstream of the green fluorescent protein (GFP) transgene cassette and substituting the neo^R cassette. The control vector (si-C) incorporated scrambled DNA sequence (Supplementary Table 1) of the same length driven by the human U6 promoter. Virus titers (vector genomes per mL) were determined by the dot blot assay (Zolotukhin et al., 1999, 2002) and were as follows: human α-syn = 9.7×10^{12}; GRP78 = 9.1×10^{12}. Titers for all rAAV-shRNA constructs were equalized to 6 x10^{12} vg/ml.

2.2. Animal experiments

Ten young (2 months; 5 male and 5 female) and 10 old (24 months; 5 male and 5 female) F344 rats (National Institute of Aging) were used to study the age-associated decline of GRP78 in the SNc. Additionally, 64 F344 female rats were used to determine if aging affects nigral DA cell survival in response to human α-syn neurotoxicity. Sixty-four young (2 months) female Sprague Dawley rats (Charles River) were used to evaluate GRP78 siRNA constructs. An additional 40 Sprague Dawley female rats (12 months) were used for the long-term GRP78 expression study. Finally, 10 young (2 months) and 10 old (24 months) female F344 rats were used to evaluate GRP78 upregulation in response to challenge with α-syn. The animals were housed at the University of Florida animal facility in a 12:12 light-dark cycle with free access to food and water. All procedures followed the National Institutes of Health guidelines and were approved by the University of Florida Institutional Animal Care and Use Committee. All surgical procedures were performed using aseptic techniques and isofluorene gas anesthesia as previously described (Gorbatyuk et al., 2008, 2010a, 2012). The rats were placed in the stereotaxic frame and injected into the SNc (anterior posterior −5.6 mm, lateral −2.4 mm from bregma, and dorsoventral −7.0 mm from dura) through a glass micropipette at a

rate of 0.5 μL/min. Animals were injected with a total of 1.5 μL for single gene or 3 μL for gene combination.

2.3. Isolation and processing of tissues

Animals were deeply anesthetized by pentobarbital injection. Brains were removed and divided into 2 parts by a coronal blade cut at approximately −3.5 mm behind the bregma. The caudal part containing the SNc was fixed in ice-cold 4% paraformaldehyde in 0.1-M phosphate buffer, pH 7.4. The fixed portions of the brains were stored overnight at 4 °C and then transferred into 30% sucrose in 0.1-M phosphate-buffered saline for cryoprotection. Forty-micrometer-thick coronal sections were cut on a freezing stage sliding microtome and processed for immunohistochemistry. The rostral piece of brain tissue was used immediately to dissect the right and left striatum. Tissue samples were weighed, frozen separately on dry ice, and kept at −80 °C until assayed. Some brains were used to obtain SNc tissue samples for Western blot analysis. Frozen brains were sectioned on a cryostat (Leica Microsystems) into 200 μm slices with SNc tissue subsequently dissected out under a microscope. Each sample was dissected individually and refrozen on dry ice for future Western blot or quantitative reverse transcription polymerase chain reaction analysis. Total tissue weight of nigral tissue obtained from 1 animal did not exceed 0.2–0.4 mg.

2.4. Immunohistochemistry

For bright-field microscopy analysis, sections were preincubated first with 1% H_2O_2–10% methanol for 15 minutes and then with 5% normal goat serum for 1 hour. Sections were incubated overnight at room temperature with anti-TH (1:2000; MAB318, mouse; Millipore) or anti-α-syn (1:1000; 61-787, mouse; BD Laboratories) antibodies. Incubation with biotinylated secondary anti-mouse antibody was followed by incubation with avidin-biotin-peroxidase complex (ABC; Vector Laboratories, Burlingame, CA, USA). Reactions were visualized using 3,3-diaminobenzidine as a chromagen.

For confocal microscopy, sections were incubated with primary antibodies against human α-syn (1:1000; 32-8100, mouse; Invitrogen), TH (1:1000; MAB318, mouse; Millipore) and GRP78 (1:400; ab-32618, rabbit; Abcam), and secondary fluorescent antibodies labeled with Alexa Fluor 488, 555, and 647 (1:500 for all; Invitrogen). The sections were examined using a Leica TCS SP5 confocal laser scanning microscope. Alexa Fluor 488 fluorescence was visualized by 488-nm excitation with a Kr/Ar laser, and emissions were examined at 500–530 nm. For imaging of Alexa Fluor 555 fluorescence, excitation at 543 nm (He/Ne laser) was used, whereas emission was observed at 560–580 nm. Alexa Fluor 647 fluorescence was induced at 633 nm (He/Ne laser) and observed at 660–700 nm. Sequential scanning was used to suppress optical crosstalk between the fluorophores in stationary-structure colocalization assays. All manipulations of contrast and illumination on color images as well as color replacement were made using Adobe Photoshop CS software.

2.5. Unbiased stereology

The unbiased stereological estimation of the total number of TH+ neurons in the SNc was performed using the optical fractionator method as previously described (Burger et al., 2004; Kirik et al., 2002). This sampling technique is not affected by tissue volume changes and does not require reference volume determinations. Sampling of cells to be counted was performed using the MicroBrightfield StereoInvestigator System. The software was used to delineate the transduction area at 5× on 40-μm sections and generate counting areas of 100 × 100 μm. A counting frame was placed randomly on the first counting area and systematically moved through all counting areas until the entire delineated area was sampled. Actual counting was performed using a 100× oil objective (Numerical Aperture 1.4). The estimate of the total number of neurons and coefficient of error were calculated according to the optical fractionator formula as previously described (Burger et al., 2004).

2.6. Immunoblotting

Tissues were suspended in 300 μL of lysis buffer (50-mM Tris, pH 7.5, 0.15-M NaCl) containing a protease inhibitor cocktail (0.1-mM PMSF, 0.5 μg/mL of Leupeptin, 0.7 μg/mL of pepstatin A) (Roche) and homogenized for 10 seconds. One hundred microliter aliquots were added immediately to 250 μL of 0.1-M $HClO_4$ and stored at −80° for subsequent measurements. The remainder of each aliquot was adjusted to a final concentration of 1% nonyl phenoxypolyethoxylethanol (NP-40), 0.1% Sodium dodecyl sulfate (SDS), incubated on ice for 30 minutes, and centrifuged for 15 minutes at 4°C. Lysates from each group were pooled and protein concentrations were determined by a Bradford assay. Fifty micrograms of each protein pool were adjusted to 2% SDS, 0.1% 2-mercaptoethanol, heated for 5 minutes at 95°C, separated on Biorad precast 4%–20% SDS-PAGE gradient gels, and transferred to polyvinylidene difluoride low-fluorescence protein membranes (PVDF-LFP; Amersham) for Western blotting. Mouse anti-α-syn (1:2000; BD Transduction Laboratories, Zymed Laboratories), mouse or rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and rabbit anti-TH (both 1:2000; Millipore) were used as recommended by the supplier. Cy5 and Cy3-conjugated goat anti-mouse and rabbit anti-goat secondary antibodies were purchased from GE Bioscience. Immunoblotted α-syn was detected and quantified with a Typhoon scanner (Amersham) by using purified α-syn-tandem affinity purification protein as a standard. Westerns used for TH quantitation included GAPDH for normalization.

2.7. Striatal DA measurements

DA samples were thawed and the equivalent of 3 mg starting tissue was diluted into 1 mL of 0.1-N $HClO_4$ containing dihydrobenzylamine as an internal control, and thoroughly homogenized. The sample was centrifuged and the supernatant was filtered through a 0.2-μm filter. DA and DOPAC (3,4-dihydroxyphenylacetic acid) levels were analyzed on a Beckman Gold System using a C18 Waters Symmetry column with an ESA and a Coulochem electrochemical detector equipped with a 5011A analytical cell. The mobile phase was composed as follows: 8.2-mM citric acid, 8.5-mM sodium phosphate monobasic, 0.25-mM EDTA, 0.30-mM sodium octyl sulfate, and 7.0% acetonitrile, pH adjusted to 3.5 then filtered through a 0.2-μm filter membrane. The flow rate was set at 1.5 mL/min with a 30-μL injection volume.

2.8. Rotational behavior

Drug-induced rotational behavior was measured after an injection of d-amphetamine sulfate (2.5 mg/kg i.p.; Sigma, St Louis, MO). Rotations were measured during a 90-minute period, and only full 360° turns were counted.

2.9. Statistical analysis

Data were analyzed using a 1-tailed unpaired *t* test and 2-way analysis of variance with Tukey's multiple comparisons post-tests using Prism 6 (GraphPad Software, Inc). Data are presented as mean ± standard error.

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3. Results

3.1. Aging affects the nigral DA cell survival in response to human α-syn neurotoxicity

To examine the possibility that overexpression of human α-syn might have a different effect on DA cell survival in old (24 months) versus young (2 months) rats, we injected rAAV expressing human α-syn into the rat SNc. Control animals were injected with rAAV containing a blank expression cassette (BV), which was an α-syn vector with an early stop codon. All of the viral vectors in this study used the rAAV5 serotype capsid. Injections were done on the right side, whereas the left was kept as an uninjected control. In this study, we used the same human α-syn rAAV stock as in our previous publication (Gorbatyuk et al., 2012), where we established that α-syn expression in the SNc at 4 weeks after vector administration was approximately 3.8-fold higher when compared with the non-injected control. Animals were killed at 1.5 and 3 months after injection. These time points were selected based on our previous findings (Burger et al., 2004; Gorbatyuk et al., 2010a, 2010b, 2012), which demonstrated that expression of α-syn reached a peak at 3–4 weeks after rAAV delivery and that neuronal degeneration in the SNc became significant after 2 months. We assumed that 3 months after injection provided sufficient time to reach a significant level of nigral degeneration in both experimental groups. The intermediate 1.5-month time point was selected to help determine if the rate of DA neuronal loss differed between young and old animals.

We first used unbiased estimation of nigral TH+ neurons to compare the rAAV-injected SNc with the uninjected SNc for each rat (Fig. 1A and B). We did not observe any significant differences between rats injected with the control BV vector at the 1.5 and 3-month time points. Animals injected with human α-syn showed a progressive loss of TH+ neurons in both young and old animals. However, this loss reached significance at the 1.5-month time point only in old animals (69% reduction compared with BV control, $p < 0.0001$; $N = 7$ for both control and experimental groups). Young rats demonstrated a 17% loss of TH+ cells, which did not reach significance versus BV (both $N = 8$). This however was dramatically less than the loss seen from human α-syn in old rats ($p < 0.001$, $N = 6$ and 8 for BV and α-syn-injected rats, respectively). At the 3-month time point, we observed a significant decrease in the number of nigral TH+ cells in both young (59% reduction) and old (85% reduction) groups of human α-syn-treated versus BV control animals. However, the human α-syn-induced effect on nigral TH+ cells in young rats was significantly less severe as compared with old animals ($p < 0.01$, $N = 7$ for both control and experimental groups).

Next, DA levels were evaluated in striatal tissues of the injected versus uninjected sides. Extracted DA was fractionated by high-performance liquid chromatography and measured by using electrochemical detection (see Section 2 for details). Our results revealed that striatal DA levels tracked with the number of surviving nigral neurons (Fig. 1C).

Old animals displayed a more severe reduction in DA pools in response to human α-syn neurotoxicity. In response to α-syn overexpression, old animals at 1.5 months after injection displayed a 48% decrease in striatal DA ($p < 0.0001$ compared with BV, both $N = 7$), which was accompanied by an ~69% loss of TH+ cells (compare Fig. 1B and C). As with the stereology count results, young rats overexpressing human α-syn demonstrated a depletion of DA that was not significant at 1.5 months as compared with the BV control (both $N = 8$) and was markedly less severe than in old animals ($p < 0.0001$; $N = 6$ for both control and experimental groups).

At 3 months after treatment, DA depletion in both young and old animals corresponded well with the number of surviving TH+ neurons. However, the degree of significance between DA depletion in young (59% reduction, $N = 8$) and old (81% reduction, $N = 6$) rats injected with human α-syn was lower ($p < 0.05$) than at the 1.5-month time point.

These results allow us to conclude that old animals demonstrate a more rapid rate of progression and a greater degree of nigral degeneration in response to human α-syn cytotoxicity.

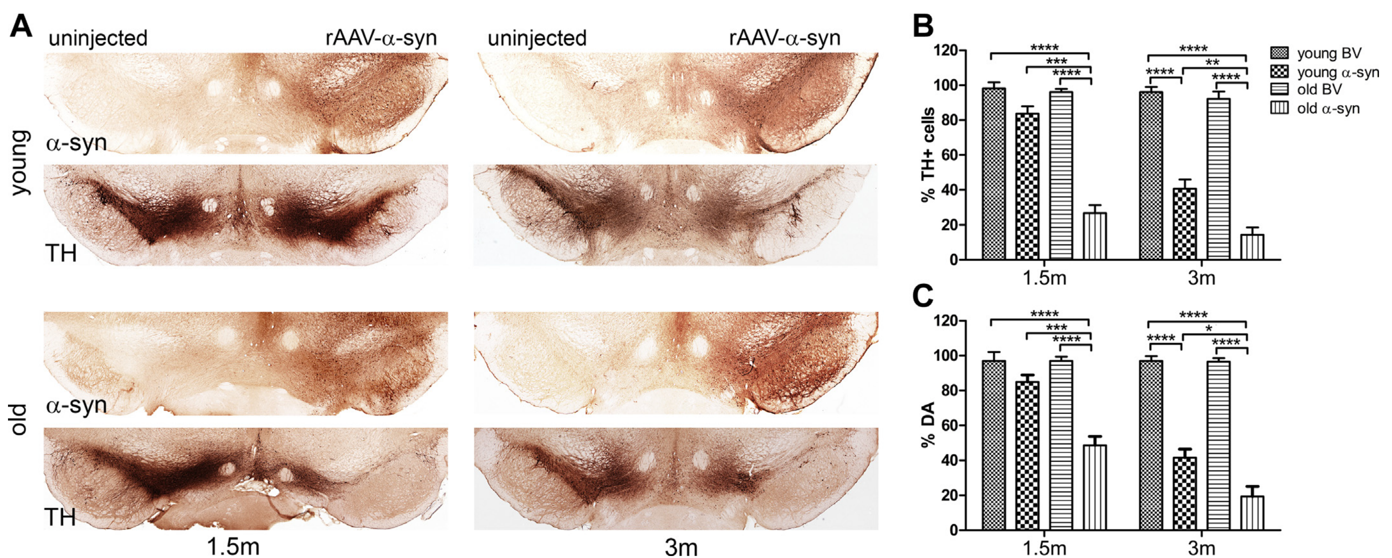

Fig. 1. Aging increases vulnerability of nigral DA cells to human α-syn neurotoxicity. This figure compares the effect of human α-syn expression on SNc neuron survival in young and old rats. (A) Bright-field photomicrographs showing nigral degeneration in representative young and old animals at 1.5 and 3 months after virus administration. Images illustrate cells immunostained for human α-syn and a reduction of TH immunoreactivity on the injected side of parallel sections. (B) Graphs showing unbiased estimation of TH+ cells remaining in the SNc and (C) quantification of striatal DA level at both time points. The percentage of TH^{+} cells and DA level were calculated by comparison with the uninjected side in the same animal. Two-way ANOVA was used. Tukey's multiple comparisons test is presented as ±SE (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; $N = 6–8$). Abbreviations: α-syn, alpha-synuclein; ANOVA, analysis of variance; BV, blank expression cassette; DA, dopamine; rAAV, recombinant adeno-associate virus; SE, standard error; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase.

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3.2. Age-associated decline of GRP78 in the rat SNc

Although an age-related reduction of GRP78 has been documented in a number of tissues, including some parts of the brain, it has not been previously demonstrated in the SNc. To determine if this phenomenon does occur in the SNc, we performed reverse transcription polymerase chain reaction and quantitative Western blot analysis on dissected nigral tissues from 2- and 24-month-old F344 rats (Fig. 2, Supplementary Fig. 1A). We did not observe significant age-related changes in mRNA expression of GRP78, PERK, or CHOP when normalized to β-actin or GAPDH (data not shown). Western blot analysis did not demonstrate any significant changes in the levels of TH but α-syn protein levels were increased by 25% only in old versus young males ($p < 0.006$; both $N = 5$). GRP78 protein levels were significantly lower in old versus young animals for both male (by 36.2%; $p < 0.02$; $N = 5$) and female (by 27%; $p < 0.02$; $N = 4$ old, 5 young) groups. These comparisons were made by normalizing to levels of GAPDH (Fig. 2, Supplementary Fig. 1B). Using β-actin as an additional house-keeping control, we confirmed the reduction in GRP78 (49%; $p < 0.001$; $N = 4$ old, 3 young) that was initially observed when comparing with GAPDH. We also observed a dramatic increase of ATF4 protein levels (201.7%; $p < 0.001$) in old versus young animals (Supplementary Fig. 1C).

3.3. Efficient expression of multiple rAAV vectors in rat SNc

To determine the efficiency of rAAV-mediated transduction in the nigrostriatal tract, we performed a preliminary experiment using 3 different single siRNAs specific to rat GRP78. These were cloned as shRNA vectors expressing both the shRNA and GFP under different promoters. Following standard procedure, we used 3 different shRNA constructs against GRP78 and a scrambled (si-C) control to avoid off-target effects. For this preliminary study, titers for all 3 rAAV-shRNA constructs and the scrambled control (si-C) (Supplementary Table 1) were equalized to 6×10^{12} μg/mL. Rats were injected unilaterally with a total of 1.5 μL of each virus, and quantitative Western blot analysis of GRP78 expression was performed with an anti-GRP78 antibody in pooled ($N = 3$) nigral tissues obtained at 4 weeks after injection. All 3 GRP78 shRNAs, but not si-C, induced a distinct reduction in target protein levels on the injected right (R) versus non-injected left (L) side in the SNc (Fig. 3A). As expected, GFP was detected only on the injected side. It is worth noting that none of the rAAV vectors used in this experiment affected nigral TH expression (Fig. 3A).

We next wanted to establish the appropriate level of human α-syn overexpression needed to reveal the difference in nigral degeneration between the animals transduced with α-syn alone or in combination with GRP78 shRNAs. Expecting that shRNAs would induce downregulation of GRP78 and thereby increase the vulnerability of nigral neurons, we decreased the α-syn vector titers to 6×10^{12} (the same as rAAV-shRNAs), thus allowing us to obtain a 2.2-fold increase in α-syn protein levels (Fig. 3B) and to increase the timescale over which we observed neurodegeneration.

We then co-injected a mixture of the rAAV-shRNA and rAAV-α-syn viruses (1.5 μL of each) into the rat SNc. The delivery of rAAV-shRNAs to the SNc was verified by detection of native GFP fluorescence (Fig. 3C). Using antibodies specific to TH, as a marker for DA neurons, and to human α-syn, we analyzed neuronal co-localization of GFP fluorescence and human α-syn at 4 weeks after injection. We found that both viruses mediated expression in the same pattern of nigral TH+ cells (Fig. 3C). However, the transduction area of GFP/α-syn neurons was not limited to the SNc because there were some GFP/α-syn-positive neurons in the pars reticulata and in the ventral tegmental area. These findings are in agreement with our previous observation (Burger et al., 2004; Gorbatyuk et al., 2008, 2012). These observations lead us to conclude that we had expressed significant amounts of human α-syn and shRNA in most of the TH+ neurons in the SNc.

3.4. siRNA-mediated downregulation of GRP78 aggravates α-syn-induced neurotoxicity in the nigrostriatal system

Two-month-old female Sprague Dawley rats were injected in the SNc with 3 μL of the appropriate mixture of 2 vectors. In control groups ($N = 6$ for each) we mixed si-1, si-2, si-3, or si-C with an rAAV vector containing a BV. This was used as a control to maintain the same volume and particle numbers for comparison with experimental groups injected with 2 viruses simultaneously. In experimental groups ($N = 10$ for each), we mixed si-1, si-2, si-3, or si-C with an rAAV vector producing human α-syn. Injections were done on one side of the brain while keeping the other side as a non-transduced internal control. We used an amphetamine-induced rotation test to monitor the same animals every 30 days for onset of behavioral deficit. At 3 months after treatment we observed a significant number of rotations toward the injected side in si-3 + α-syn experimental group of animals, as compared with control rats (see below). We did not observe statistically significant changes in the behavior test of the other experimental groups, including rats injected with si-C + α-syn. At this point, the experiment was stopped and the animals were killed for further analysis.

Confocal microscopy revealed visually discernible differences between experimental and control animals (Supplementary Fig. 2). To give these results context, the degree of reduction in GRP78 protein was estimated in control rats injected with si-1, si-2, si-3, or si-C, combined in each case with BV. For this, the nigral sections were immunostained for GRP78 and TH (Fig. 4A), and the level of GRP78 staining was estimated in GFP-positive nigral neurons that co-stained for TH on the injected side. These measurements were

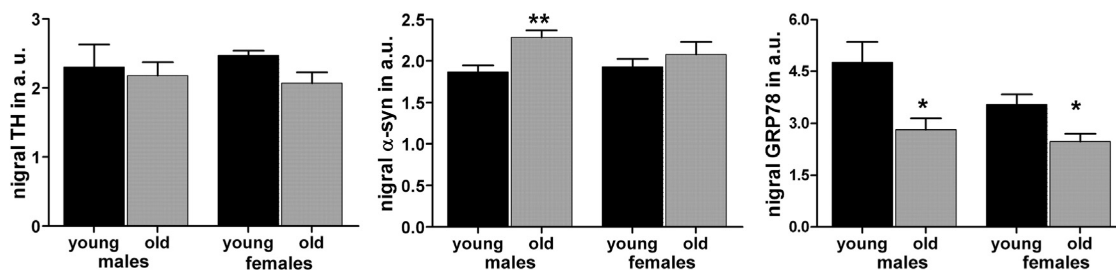

Fig. 2. Downregulation of GRP78 expression accompanies aging in the rat SNc. Nigral tissue extracts from young (2 months) and old (24 months) rats were Western blotted with GRP78, TH, α-syn, and GAPDH antibodies (see Supplementary Fig. 1B). The amount of analyzed proteins was normalized to GAPDH and shown on graphs in arbitrary units (a.u.). One-tailed unpaired t test. Data are presented as mean ± SE (*$p < 0.05$, **$p < 0.01$; $N = 4$ young females; 5 all other groups). Abbreviations: α-syn, alpha-synuclein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase, GRP78, glucose regulated protein 78; SE, standard error; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase.

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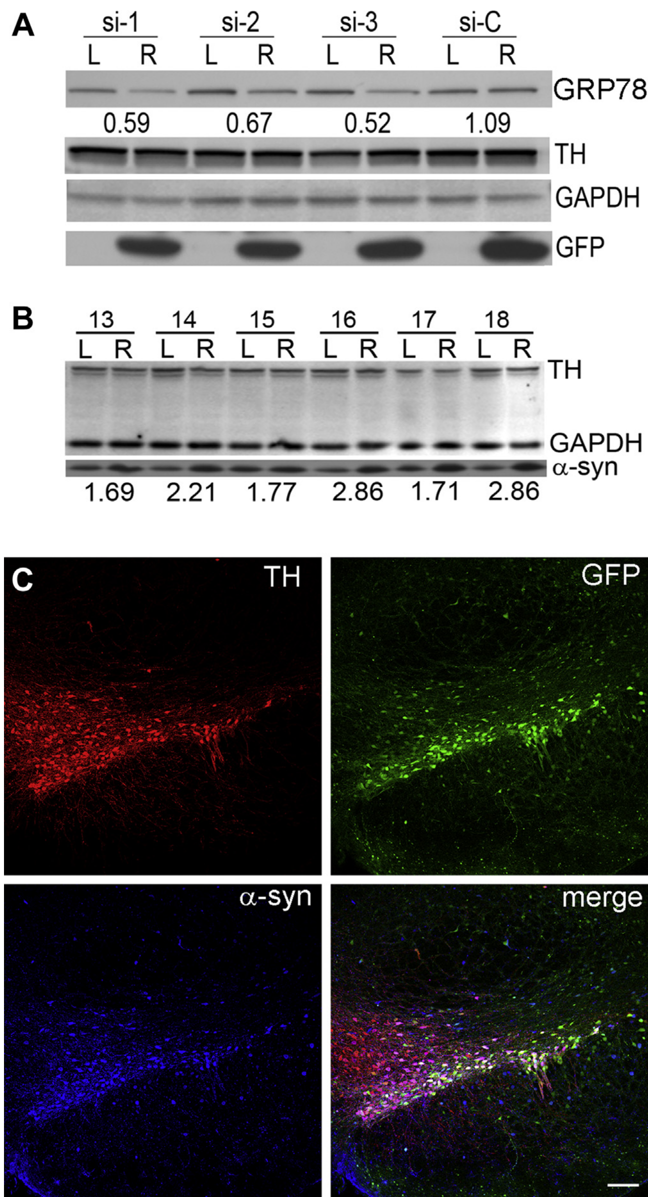

Fig. 3. GRP78 expression can be augmented with specific shRNAs. Expression of GRP78 shRNA (A) and human wt α-syn alone (B) and together (C) in the SNc at 1 month after rAAV injection. (A) Comparison of 3 different shRNAs (si-1, si-2, si-3) against rat GRP78 and a scrambled control shRNA (si-C) in the SNc of rats at 1 month after vector injection. All 4 vectors also express GFP. Pooled ($N = 3$ for each group) nigral tissue extracts were Western blotted with GRP78, TH, and GAPDH antibodies. The amount of GRP78 was normalized to GAPDH and the ratio of the injected (R) versus uninjected (L) sides was calculated (I/U). Note there was no loss of TH-positive staining in any of the groups at this time point. (B) α-Syn protein level in the SNc at 1 month after vector injection. Nigral tissue was excised at 1 month after injection from uninjected (L) and injected (R) sides of individual animals (13–18) that had been treated with rAAV-human α-syn. Nigral tissue extract was Western blotted with anti-α-syn antibody that equally recognizes rat and human α-syn (Gorbatyuk et al., 2012). The ratio of α-syn on the injected and uninjected sides (I/U) was calculated for each animal. Note there was no loss of TH-positive staining in any of the animal groups at this time point. (C) Confocal images demonstrate co-expression of both GFP (green) (expressed by the GRP78 shRNA vectors) and human wt α-syn (blue) in TH-positive (red) neurons of the SNc injected with both viruses simultaneously (bar = 150 μm). Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; GRP78, glucose regulated protein 78; shRNAs, small hairpin RNA; α-syn, alpha-synuclein; SNc, substantia nigra pars compacta; rAAV, recombinant adeno-associate virus; TH, tyrosine hydroxylase. (For interpretation of the references to color in this Figure, the reader is referred to the web version of this article.)

repeated and compared for both the injected and uninjected sides of the brain. There was a substantial reduction of GRP78 in injected versus non-injected nigras. Using confocal image analysis software (Leica Applications Suite, Leica Microsystems CMS GmbH, Mannheim, Germany), it was determined that the level of GRP78 staining in TH+ neurons was 62% of the control side in animals injected with si-1 ($p < 0.001$), 65% for those injected with si-2 ($p < 0.05$), and 51% in animals injected with si-3 ($p < 0.0001$; $N = 4$ for all groups, 300 cells/group). No significant changes in GRP78 were seen in rats injected with si-C.

Next, striatal tissues from all animals were Western blotted to determine the levels of TH on the injected right (R) versus uninjected left (L) side (Supplementary Fig. 3). We found that all experimental groups containing animals injected with α-syn and shRNAs, but not α-syn and si-C, demonstrated significant reduction in striatal TH protein levels, which was most prominent in rats injected with si-3 + α-syn (Fig. 4B). Rats, which were injected with a mixture of α-syn and si-1, si-2, or si-3, retained 72.1 ± 3.0%, 62.9 ± 8.7%, or 52 ± 7.8% of the TH protein seen on the uninjected side, respectively. This was in contrast to the more modest, nonsignificant reduction in TH (80.7 ± 8.7%) seen for the α-syn + si-C injection. In contrast, the level of TH in the striatum of control rats injected with BV and si-1, si-2, or si-3 was not significantly reduced ($N = 10$ for experimental and $N = 6$ for control groups).

Unbiased stereology counts of the number of TH+ cells in the SNc (Fig. 4C) were done to quantitate the extent of neurodegeneration. We found that lowering GRP78 with si-1, si-2, and si-3 significantly exacerbated nigral degeneration in response to human α-syn expression (Fig. 4B). Among the rAAV-shRHAs, si-3 was more potent at aggravating α-syn neurotoxicity using the same input genome dose as si-1 and si-2. This was consistent with its higher knockdown activity against GRP78 as determined by WB in nigral tissues at 4 weeks after injection (Fig. 3A) and by confocal image analysis of control groups at 3 months after treatment (Fig. 4A). There was no significant TH+ cell loss identified in the α-syn + si-C-injected SNc (Fig. 4B).

The measurement of striatal DA levels in all experimental groups ($N = 6$) agreed with TH protein and stereology count results. The α-syn + si-3-injected rats demonstrated dramatic DA depletion in the striatum compared with both control BV+si-3 and α-syn + si-C-injected animals (Fig. 4C).

The loss of TH+ neurons in the SNc and striatal DA was accompanied by a significant amphetamine-induced rotational asymmetry in rats injected with α-syn + si-3 (Fig. 4C). All other experimental groups injected with α-syn displayed ipsilateral rotation (toward the injected side); however, those results did not reach statistical significance (Fig. 4C bottom graph). This is consistent with previous observations that significant impairment in drug-induced and spontaneous motor behavior would appear only in animals which experienced a >50%–60% loss of nigral DA neurons or striatal DA levels (Kirik et al., 1998, 2002).

3.5. *Aging affects the nigral cell capacity to upregulate endogenous GRP78 protein in response to α-syn neurotoxicity*

To evaluate if ER stress induced by human α-syn in the SNc leads to equal upregulation of GRP78 in young (2 months) and old (24 months) rats, we injected rAAV expressing human α-syn on the right side of the brain in both groups. rAAV with BV was administered into the SNc on the contralateral side as control virus injection. Quantitative Western blot analysis of GRP78, TH, and α-syn expression was performed on dissected nigral tissues of individual animals at 4 weeks after injection (Fig. 5, Supplementary Fig. 4). As expected, no human α-syn expression was detected on the control

left side. The level of total rat and human α-syn expression in the nigral tissue 4 weeks after injection with wild-type α-syn vector was approximately 4.1 (young) and 3.8 (old)-fold higher than the control side of the brain. In addition, there was no significant difference between the levels of TH protein when normalized to total protein. However, comparative analysis of nigral tissues obtained from old and young rats revealed that aging affected the capacity of nigral cells to upregulate endogenous GRP78 protein in response to human α-syn expression. We identified a significant (up to 152%; $N = 4$, $p < 0.01$) upregulation of GRP78 protein in the injected SNcs compared with the control sides of young rats. At the same time, overexpression of human α-syn in the SNc of old rats did not induce a significant upregulation of GRP78 (~121%, $N = 4$, $p > 0.05$).

3.6. Sustained overexpression of GRP78 protects aging nigral neurons against α-syn neurotoxicity

To test the hypothesis that a prolonged increase in GRP78 could be therapeutically beneficial for aging nigral DA cells, we injected rAAV vectors in the SNc of 12-month-old female Sprague Dawley rats to overexpress GRP78 and human α-syn simultaneously. We also performed injections with each virus separately. BV was once again used to maintain a consistent volume and particle number between single and double vector injections and was used as a virus injection control. In this study, we used the same virus titers for human α-syn and GRP78 as we used in our previous publication (Gorbatyuk et al., 2012) in which the level of total α-syn expression in the SNc at 4 weeks after vector administration was approximately 3.8-fold higher compared with the control non-injected side. At the same time, GRP78 protein in the injected SNc was upregulated 1.4-fold compared with the non-injected side (Gorbatyuk et al., 2012). We had previously shown that the cotransduction with human α-syn and tagged GRP78 constructs did not alter protein expression, as compared with individual injections. The exogenous (tagged) GRP78 was also shown to co-immunoprecipitate with human α-syn, suggesting that it is indeed active (Gorbatyuk et al., 2012).

Amphetamine rotation tests were performed at 1, 3, 6, and 9 months after vector administration (Fig. 6A) and revealed a notable number of rotations toward the injected side only in human α-syn-overexpressing rats. It reached significance at 3 months after injection compared with BV, α-syn+GRP78, or GRP78-injected animals (accordingly, 423.7 ± 144.1 vs. −1.8 ± 9.7, $p < 0.01$; −91.7, $p < 0.001$ or 17.6 ± 48.4, $p < 0.001$; $N = 9$ for all groups) and continued to demonstrate a significant number of ipsilateral rotations at 6- and 9-month time points compared with all control and experimental animal groups.

Rats were sacrificed 4 days after the last rotation test, when they were 21 months old. Rats injected with GRP78 showed higher expression of the chaperone in the SNc on the right side (Fig. 6B). Using the same confocal image analysis software approach as we used in the previously mentioned shRNAs study, we determined that the level of GRP78 staining in TH+ neurons (Fig. 6B) in the injected SNc was 2.1-fold higher than the control side ($p < 0.001$). No significant change was seen in GRP78 levels in animals injected with control BV ($N = 4$ for both groups, 300 cells/group).

As expected, immunoblotting of striatal tissue extracts with human α-syn antibody revealed expression of human α-syn only on the injected side. Examination with an anti-TH antibody demonstrated severe loss of TH protein (25.8 ± 6.6%, $N = 9$) in the striatum of animals overexpressing human α-syn only (Fig. 6C, Supplementary Fig. 5). The level of TH protein was significantly lower compared with control BV and GRP78-injected groups (for both $N = 8$, $p < 0.001$). Co-expression of human α-syn with exogenous GRP78 significantly increased striatal TH protein (59.3 ± 7.3%, $N = 9$; $p < 0.001$) compared with single human α-syn overexpression. However, this level of expression was still significantly lower compared with both BV and GRP78 groups of animals ($p < 0.01$).

In parallel with TH protein measurements, we also assayed striatal DA in rAAV-injected animals (Fig. 6C). Striatal DA was diminished in rats injected with human α-syn compared with animals injected with control BV (21.3 ± 5.2, $N = 7$ vs. 102.7 ± 4.2%, $N = 5$; $p < 0.001$) and with GRP78 (21.3 ± 5.2% vs. 104.2 ± 5.7%, $N = 6$; $p < 0.001$). Co-injection of GRP78 with human α-syn significantly ameliorated the decline of striatal DA compared with animals injected with human α-syn alone (70.5 ± 4.7%, $N = 7$ vs. 21.3 ± 5.2%; $p < 0.001$). Overexpression of GRP78 alone did not affect DA levels in the striatum compared with control BV injections (104.2.5 ± 5.7% vs. 102.7 ± 4.2%, $p > 0.05$).

Unbiased estimation of nigral TH+ cells revealed that overexpression of human α-syn caused a severe loss of neurons in the SNc at 9 months after injection (Fig. 6C and D). We determined that the number of TH+ cells was reduced dramatically (76%) in animals overexpressing human α-syn compared with BV control (24 ± 6.3%, $N = 9$ vs. 97.7 ± 3.4%, $N = 8$; $p < 0.001$). Co-expression of GRP78 with human α-syn prevented much of the loss of TH+ neurons caused by human α-syn (24 ± 6.3%, $N = 9$ vs. 69.4 ± 6.3%, $N = 9$; $p < 0.001$). However, these animals exhibited a 28% loss of TH+ cells compared with BV, which was significant ($p > 0.01$). GRP78 overexpression alone did not affect the number of nigral TH+ neurons. Likewise, in our previous study (Gorbatyuk et al., 2010b), we co-expressed α-syn with phospholipase D2 or GFP and found that neither protein had a protective effect unlike GRP78. Moreover, phospholipase D2 possessed a more pronounced toxicity compared with α-syn.

4. Discussion

The results outlined in this study suggest that a decline in GRP78 expression during aging increases vulnerability of nigral DA neurons to human α-syn cytotoxicity and might be a predisposing factor for the onset and progression of synucleinopathies in humans. Our results demonstrate a correlation between the level of GRP78 knockdown provided by each siRNA and the level of neurodegeneration seen in our samples, making off-target effects highly unlikely as the cause of our observed results. Our findings of a significant loss of GRP78 protein in the SNc of old animals echo previous findings that have described a similar phenomenon in other tissues (Erickson et al., 2006; Nuss et al., 2008). This loss of GRP78 does not extend to the mRNA level, suggesting that GRP78 protein levels are controlled either on a translation level or via increased protein turnover.

GRP78 is a multifunctional chaperone that facilitates the refolding or proteasomal degradation of misfolded proteins and serves as a regulator of UPR signaling (Lee, 2005; Naidoo and Brown, 2012). It is through these roles that GRP78 imparts some of its antiapoptotic activity as it serves to resolve conditions that lead to long-term ER stress, such as accumulation of misfolded proteins, and the associated late-stage, proapoptotic UPR signaling (Naidoo and Brown, 2012; Pfaffenbach and Lee, 2011). Conversely, loss of GRP78 activity reduces the cellular capacity to maintain healthy protein folding and to deal with stress, thus shifting the intracellular environment toward a proapoptotic balance (Li et al., 2014). Indeed our shRNA results indicate that loss of GRP78 activity sensitizes the cells to stress, which in our case was provided by the overexpression of human α-syn. The fact that the 3 tested siRNAs caused differing degrees of GRP78 knockdown proved to be instructional, because it revealed that a greater reduction of GRP78 corresponded with greater α-syn toxicity. This relationship was observed at the molecular, cellular, and behavioral levels (Fig. 4B).

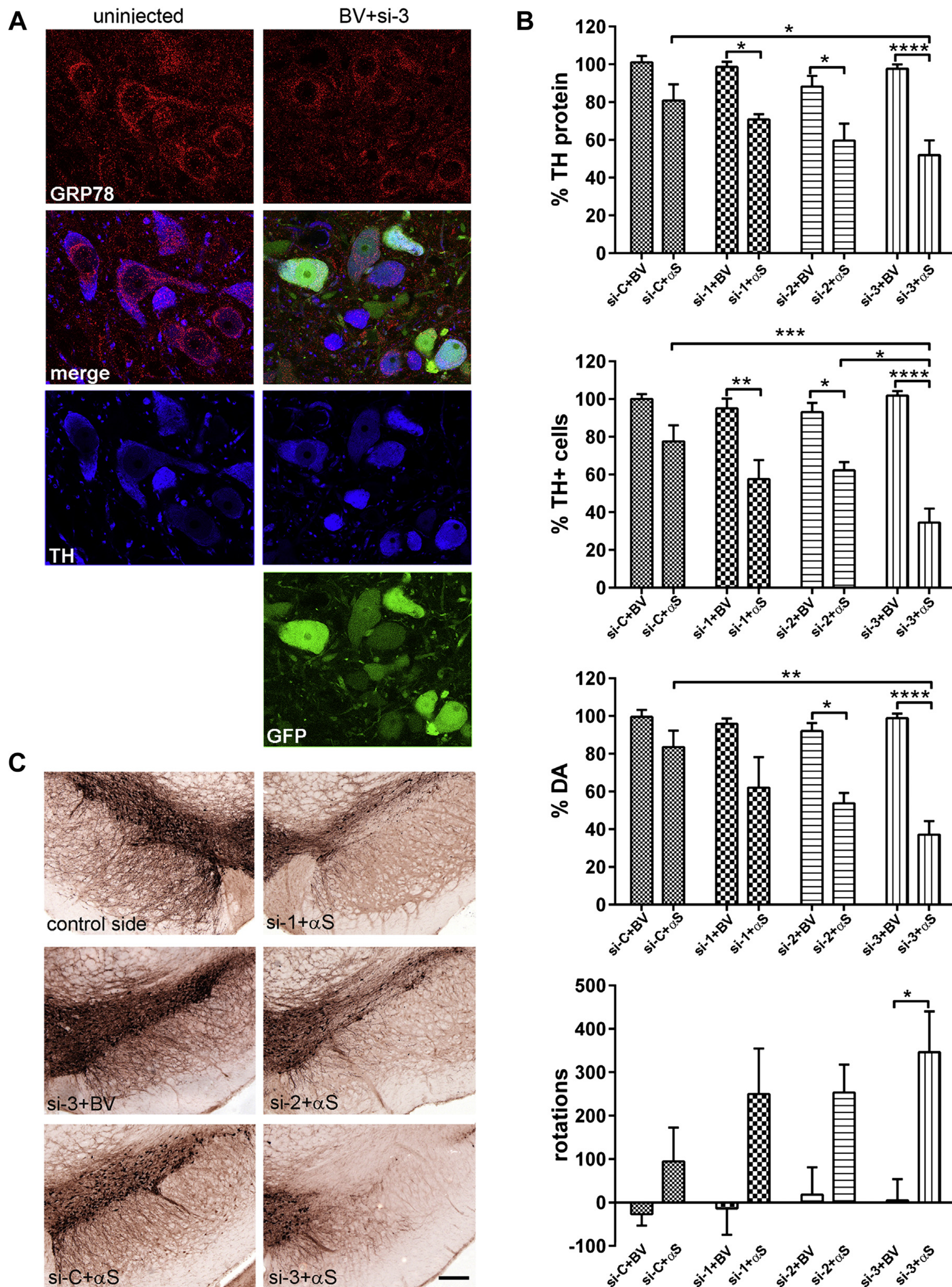

Fig. 4. Degree of decline in GRP78 expression correlates with striatal TH protein levels, nigral DA cell survival, and amplitude of behavioral asymmetry in response to human α-syn (αS) overexpression. (A) GRP78 protein expression level in DA neurons of the SNc at 3 months after rAAV-GRP78 shRNA injection. Confocal images show GRP78 (red) and TH (blue) as well as native GFP (green) in nigral neurons at 3 months after combined injection of viruses expressing si-3 and BV. TH is used as a marker for DA cells. Images illustrate protein expression levels of GRP78 (red) in nigral TH+ neurons (blue) on non-injected (left panel) and injected (right panel) sides. (B) The effect of GRP78 decline and human wt α-syn expression on striatal TH protein levels (upper graph), unbiased estimation of nigral TH+ neuron survival as well as striatal DA level and rotational behavior (bottom graph) at 3 months after rAAV injection. Striatal TH-protein level and the number of nigral TH+ cells remaining on the injected side shown as a percentage of the uninjected side ±SE. Amount of striatal TH protein, DA, and number of nigral TH+ neurons were most dramatically reduced in si-3 + αS-injected rats compared with control si-3 + BV and with si-C + αS (and si-2 + αS for TH cells count)-injected animals. The amphetamine-induced rotation test revealed a significant number of ipsilateral rotations only in rats injected with si-3 + αS versus control si-3 + BV animals. Two-way ANOVA analysis. Tukey's multiple comparisons test is indicated as *, **, ***, **** = $p < 0.05$, 0.01, 0.001, and 0.0001, respectively, versus

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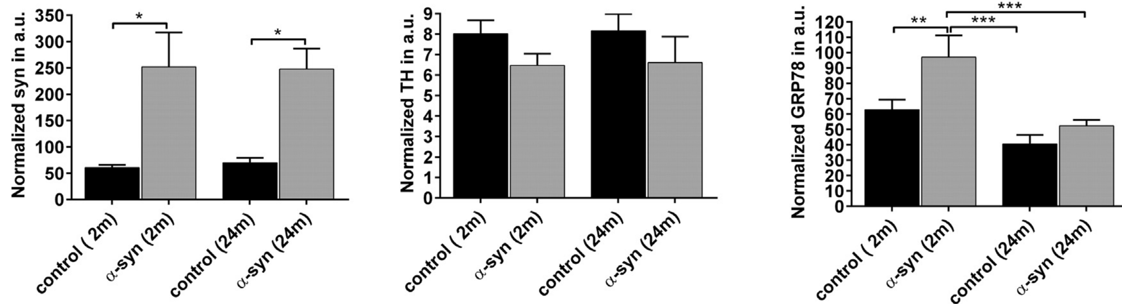

Fig. 5. Aged nigral cells have a reduced capacity to upregulate endogenous GRP78 in response to human α-syn overexpression. Graphs show the level of TH, GRP78, or total α-syn expression in rAAV-human α-syn injected side compared with control rAAV-BV administered in young (2 months) and old (24 months) animals (see immunoblot images in Supplementary Fig. 4). Each graph shows the change induced in each of the 3 proteins in response to human α-syn neurotoxicity. The amount of detected proteins was normalized to GAPDH. Two-way ANOVA analysis. Tukey's multiple comparisons test is indicated as *, **, *** = $p < 0.05$, 0.01, and 0.001; $N = 4$ (for TH and GRP78) and $N = 3$ (for α-syn) per group. Abbreviations: α-syn, alpha-synuclein; ANOVA, analysis of variance; BV, blank expression cassette; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GRP78, glucose regulated protein 78; rAAV, recombinant adeno-associate virus; TH, tyrosine hydroxylase.

An age-associated rise in the level of α-syn was previously reported (Kahle et al., 2000) and, taken together with our findings, could explain why pathology onset is often later in life, when neurons are in a more vulnerable state by virtue of worn-down cellular machinery. With this said, it needs to be confirmed by future clinical studies whether the increase in α-syn is an independent phenomenon, or if it stems from the age-related decrease in GRP78 activity, which would leave the cell progressively less able to degrade misfolded α-syn. Indeed a major component of Lewy bodies, a hallmark of PD, is phosphorylated α-syn (Cookson, 2005, 2009). This could simply be protein that was initially marked for proteasomal degradation, but instead because of aging cellular machinery became sequestered in aggregates in an attempt to minimize harm to the cell.

Our own data demonstrated that older animals were less able to respond to challenge with α-syn (Fig. 5). However, it is not clear if the subsequent cell loss was due to acute α-syn toxicity or to long-term activation of the UPR. Perpetual UPR activation caused by ever-increasing amounts of aggregated protein could lead to cell death through apoptosis: a well-characterized consequence of unresolved ER stress (Muchowski and Wacker, 2005).

Age-associated deficits in a number of other chaperones and the proteasome have been previously described (Nuss et al., 2008), revealing a template for a broad range of age-related degenerative diseases. These diseases could vary in their localization and/or the main protein constituent comprising the aggregates (α-syn, β amyloid, and so forth) but may fundamentally be the result of the same age-related breakdown of the cell's capacity to deal with misfolded proteins. A gradual, age-related loss of ER function would thus condemn cells to create a backlog of misfolded proteins, which they could no longer clear. In failing to resolve the ER stress, long-term UPR signaling would lead to apoptosis. In this light, it is conceivable that the particular degenerative disease an individual would develop would be heavily influenced by the stresses endured in a particular tissue. Indeed, a lifetime of oxidative damage may very well result in ER stress, cell death, and physiological deficits by the mechanism outlined above.

Fortunately, the emerging picture of the molecular events that may underlie the development and onset of PD also points to a potential therapeutic approach. We have previously shown that rAAV-mediated upregulation of GRP78 could be neuroprotective when challenging 2-month-old rats with overexpression of human α-syn in the SNc. We found that GRP78 significantly attenuated the ER stress response by diminishing upregulation of mediators of 2 UPR signaling pathways: ATF6 and PERK (Gorbatyuk et al., 2012). A downregulation of the latter was very recently shown to prevent neurodegeneration, as well as accumulation and aggregation of misfolded proteins, in prion-infected mice (Moreno et al., 2013). Although the findings of those studies in young rats were interesting, GRP78 would need to be effective in older animals to show real therapeutic promise. Taking into account our current data and results of others (Kim et al., 2011) showing that aged nigral neurons are more vulnerable to human α-syn cytotoxicity, our present study has provided the preliminary evidence that long-term upregulation of GRP78 appears to be safe in our animal model. Comparing the obtained results in the animal model with human pathology, this is important because PD symptoms do not manifest until at least 60%–80% of the DA neurons in the SNc are already lost. Baring the development of a reliable test for the early stages of this process, prophylactic treatment could prove to be an effective course of action in preventing pathology versus simply treating disease symptoms.

Although rAAV-mediated gene transfer has proven to be an invaluable research tool, it is not the only approach to elevating GRP78 expression. Alternative approaches include the use of 2-deoxy-D-glucose (2-DG; a non-metabolizable analogue of glucose; tissue culture only) (Duan and Mattson, 1999), a selective inducer of GRP78/BiP, BiP Inducer X (Oida et al., 2010), methoxyflavones (Takano et al., 2007), and histone deacetylase inhibitors (Hoozemans et al., 2012). Treatment with histone deacetylase inhibitors has been shown as an effective way to boost GFP78 expression and activity of existing GRP78 protein (Chen et al., 2012; Moreno et al., 2013).

Future studies will need to focus on these or other pharmacological means of altering GRP78 expression and activity as well as the potential effects of GRP78 on α-syn-induced neuropathology, such as inclusions and axonal swelling. We believe these results show promise for the next generation of therapeutics aimed at the treatment and prevention of synucleinopathies, including PD.

Disclosure statement

The authors have no conflicts of interest to disclose.

appropriate control rats; $N = 6$ (for control groups) and 10 (for experimental groups). (C) Bright-field photomicrographs showing remaining TH-positive cells in the SNc of representative animals from different experimental groups. Bar = 250 μm. Abbreviations: α-syn, alpha-synuclein; BV, blank expression cassette; DA, dopamine; GFP, green fluorescent protein; GRP78, glucose regulated protein 78; rAAV, recombinant adeno-associate virus; SE, standard error; shRNA, small hairpin RNA; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase. (For interpretation of the references to color in this Figure, the reader is referred to the web version of this article.)

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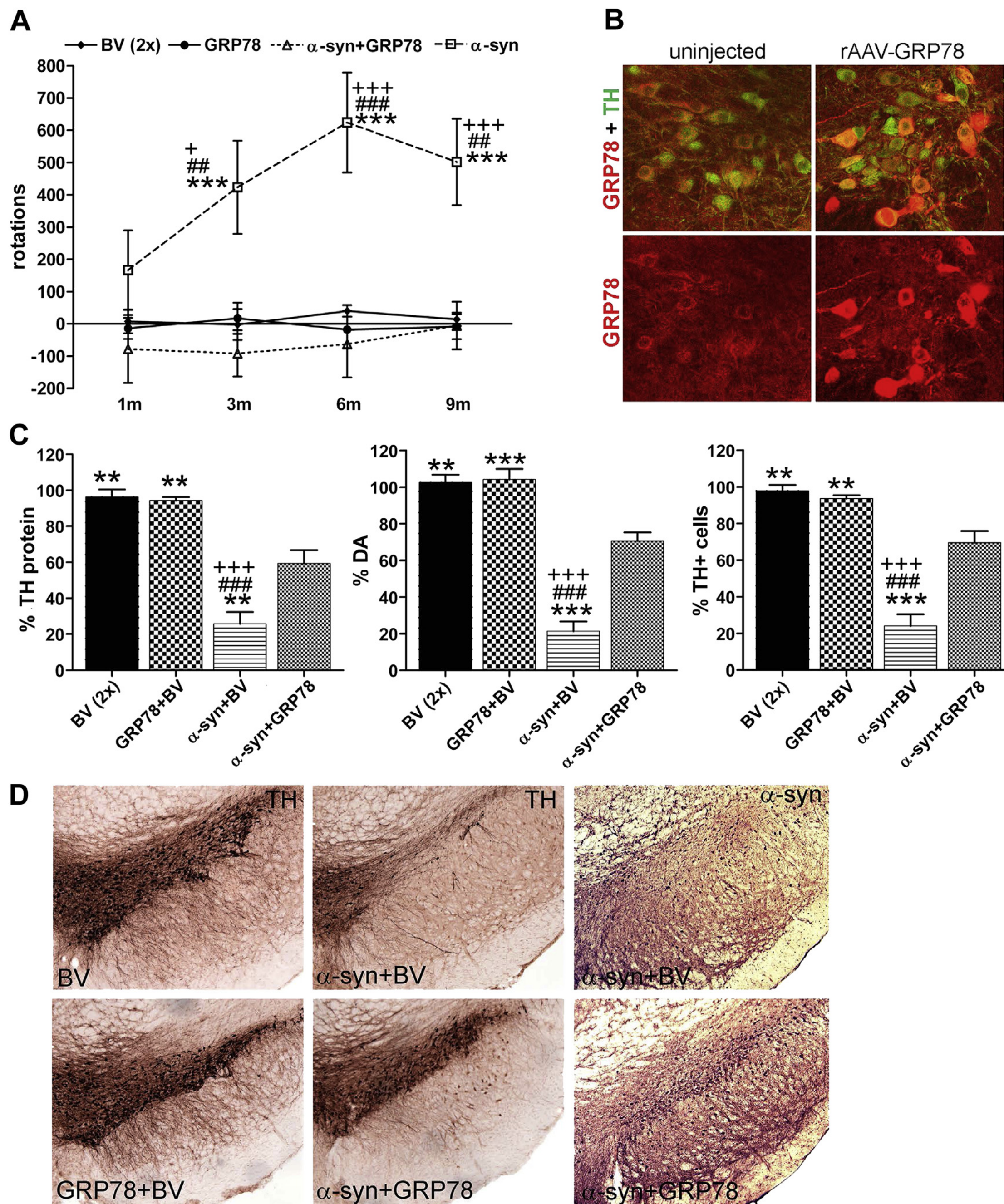

Fig. 6. Overexpression of GRP78 in aging nigral DA neurons diminishes α-syn-induced neurodegeneration. (A) Amphetamine-induced rotation test versus time after rAAV injections. Starting at 3 months a significant number of rotations toward the injected side (positive, ipsilateral rotation) was revealed only in α-syn-injected rats compared with all other animal groups. Amphetamine-induced rotation was measured for 90 minutes and the average rotation +SE is shown. Two-way ANOVA analysis. Tukey's multiple comparison test is indicated as $^{+}$, $^{\#\#}$, and $^{+++}$ ($^{\#\#\#}$ or ***) $= p < 0.05$, 0.01, and 0.001 versus BV ($^{\#}$), GRP78 ($^{+}$), and α-syn plus GRP78 (*); $N = 9$ per group. (B) GRP78 protein expression in DA neurons of the SNc at 9 months after AAV-GRP78 shRNA injection. Confocal images illustrate protein expression level of GRP78 (red) in nigral TH-positive (green) neurons on non-injected (left panel) and injected (right panel) sides. (C) Quantitate analysis of striatal TH protein revealed by Western blot (left graph, see Supplementary Fig. 5). The graph shows the level of TH protein remaining in the striatum on the injected side as a percentage of the uninjected side ±SE at 9 months after injection. Two-way ANOVA analysis. Tukey's multiple comparison test is indicated as ** and *** ($^{\#\#\#}$ or $^{+++}$) $= p < 0.01$, and 0.001, respectively, versus BV ($^{\#}$), GRP78 ($^{+}$), and α-syn plus GRP78 (*); $N = 8$–9 per group. Middle graph shows loss of DA in the striatum injected with a vector expressing α-syn. Overexpression of GRP78 protein along with α-syn significantly (by 49%) preserved DA levels as compared with α-syn injected alone. This was also significantly different from the BV control. Overexpression of GRP78 protein alone did not have an effect on DA in injected SNcs compared with control BV injection. Two-way ANOVA analysis. Tukey's multiple comparison test is indicated as ** and *** ($^{\#\#\#}$ or $^{+++}$) $= p < 0.01$ and 0.001, respectively, versus BV ($^{\#}$), GRP78 ($^{+}$), and α-syn plus GRP78 (*); $N = 5$ (GRP78 control) − 7 (all others) per group. The right graph shows unbiased estimation of TH-positive cells remaining in SNc on the injected side as a percentage of the uninjected side ±SE at 9 months after injection. The number of TH-positive neurons counted in the α-syn-injected SNc was dramatically reduced compared with control BV rats. Co-expression of α-syn and GRP78 proteins led to significant rescue of TH-positive cells preventing cell death compared with a single human α-syn injection. However, these animals demonstrate a 28% and 24% loss of TH-positive cells compared with BV and GRP78 animals, respectively, which is significant in both cases. GRP78 overexpression alone did not reduce the number of TH-positive cells. Two-way ANOVA analysis. Tukey's multiple comparison test is indicated as ** and *** ($^{\#\#\#}$ or $^{+++}$) $= p < 0.01$ and 0.001, respectively, versus BV ($^{\#}$), GRP78 ($^{+}$), and α-syn plus GRP78 (*); $N = 8$–9 per group. (D) Bright-field photomicrographs showing remaining TH-positive cells and human α-syn immunostaining in the SNc of representative animals from different experimental groups. Abbreviations: α-syn, alpha-synuclein; ANOVA, analysis of variance; BV, blank expression cassette; DA, dopamine; GRP78, glucose regulated protein 78; rAAV, recombinant adeno-associate virus; SE, standard error, shRNA, small hairpin RNA; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase. (For interpretation of the references to color in this Figure, the reader is referred to the web version of this article.)

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Acknowledgements

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2015.02.018.

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