doi:10.1093/brain/awt131 Brain 2013: 136; 2130–2146 | 2130

BRAIN
A JOURNAL OF NEUROLOGY

Boosting chaperone-mediated autophagy *in vivo* mitigates α-synuclein-induced neurodegeneration

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α-Synuclein levels are critical to Parkinson's disease pathogenesis. Wild-type α-synuclein is degraded partly by chaperone-mediated autophagy, and aberrant α-synuclein may act as an inhibitor of the pathway. To address whether the induction of chaperone-mediated autophagy may represent a potential therapy against α-synuclein-induced neurotoxicity, we overexpressed lysosomal-associated membrane protein 2a, the rate-limiting step of chaperone-mediated autophagy, in human neuroblastoma SH-SY5Y cells, rat primary cortical neurons *in vitro*, and nigral dopaminergic neurons *in vivo*. Overexpression of the lysosomal-associated membrane protein 2a in cellular systems led to upregulation of chaperone-mediated autophagy, decreased α-synuclein turnover, and selective protection against adenoviral-mediated wild-type α-synuclein neurotoxicity. Protection was observed even when the steady-state levels of α-synuclein were unchanged, suggesting that it occurred through the attenuation of α-synuclein-mediated dysfunction of chaperone-mediated autophagy. Overexpression of the lysosomal receptor through the nigral injection of recombinant adeno-associated virus vectors effectively ameliorated α-synuclein-induced dopaminergic neurodegeneration by increasing the survival of neurons located in the substantia nigra as well as the axon terminals located in the striatum, which was associated with a reduction in total α-synuclein levels and related aberrant species. We conclude that induction of chaperone-mediated autophagy may provide a novel therapeutic strategy in Parkinson's disease and related synucleinopathies through two different mechanisms: amelioration of dysfunction of chaperone-mediated autophagy and lowering of α-synuclein levels.

Keywords: alpha-synuclein; chaperone-mediated autophagy; Lamp2a; neurotoxicity; Parkinson's disease

Abbreviations: AAV = adeno-associated virus; CMA = chaperone mediated autophagy; Lamp2a = lysosomal-associated membrane protein 2a; TH = tyrosine hydroxylase

Received December 30, 2012. Revised April 1, 2013. Accepted April 2, 2013. Advance Access publication June 11, 2013

Introduction

Parkinson's disease is an age-related neurodegenerative disorder with unknown aetiology. The pathological hallmarks of both familial and sporadic Parkinson's disease are Lewy bodies, proteinaceous inclusions seen in the affected regions of post-mortem parkinsonian brains that stain strongly for the presynaptic protein, α-synuclein. Growing evidence from genetic (Nalls *et al.*, 2011), pathological (Spillantini *et al.*, 1997), animal model (Magen and Chesselet, 2010; Ulusoy *et al.*, 2010; Low and Aebischer, 2012) and biochemical studies (Cookson, 2005) strongly support the theory that aberrant α-synuclein plays a critical role in familial and sporadic Parkinson's disease pathogenesis.

The basic characteristic of α-synuclein is its tendency to form oligomers and aggregates, which is influenced by the amount of α-synuclein, giving rise to the 'increased α-synuclein burden' hypothesis. According to this hypothesis, the critical factor in idiopathic Parkinson's disease pathogenesis is α-synuclein accumulation, either because of enhanced transcription or through reduced degradation (Kim and Lee, 2008). Thus, it is important to uncover ways of controlling α-synuclein protein load to combat its neurotoxicity and, therefore, elucidating the machinery responsible for its turnover is a prime target for therapy.

The manner of α-synuclein degradation in neurons remains controversial. Studies from our group and others showed that wild-type α-synuclein is degraded mainly through chaperone-mediated autophagy (CMA) (Cuervo *et al.*, 2004; Vogiatzi *et al.*, 2008) and macroautophagy (Vogiatzi *et al.*, 2008). The Parkinson's disease-linked mutants A53T and A30P cannot be degraded through CMA, but instead bind strongly to the CMA receptor, lysosomal-associated membrane protein 2a (Lamp2a) and impair CMA (Cuervo *et al.*, 2004; Xilouri *et al.*, 2009). Importantly, post-translational modifications of the wild-type protein also impact CMA degradation (Martinez-Vicente *et al.*, 2008; Xilouri *et al.*, 2009). In particular, the inhibitory effect of dopamine-modified wild-type α-synuclein on CMA could be an underlying explanation for the higher rates of degeneration observed in dopaminergic neurons in Parkinson's disease. Nevertheless, the importance of CMA in α-synuclein degradation, especially in the living brain, is not clear-cut (for a review see Xilouri *et al.*, 2013). Mak *et al.* (2010) found that the induction of CMA activity correlated with enhanced α-synuclein clearance in the mouse substantia nigra, although a direct causal relationship was not established. Another *in vivo* study found that lysosomal-dependent α-synuclein degradation occurred only with excess α-synuclein burden in the mouse cortex, whereas proteasomal degradation was important for the clearance of endogenous and overexpressed α-synuclein (Ebrahimi-Fakhari *et al.*, 2011). Ageing is the prime risk factor for Parkinson's disease (Hindle, 2010; Surmeier *et al.*, 2010); intriguingly, CMA declines with age (Cuervo and Dice, 2000*c*) and α-synuclein protein levels are increased in the aged substantia nigra of humans and monkeys (Chu and Kordower, 2007), which is associated with dopaminergic dysfunction, indicating that CMA dysfunction may be one of the factors involved in the association of Parkinson's disease with ageing.

The main cause for CMA decline during ageing is the reduced lysosomal abundance of Lamp2a, the rate-limiting step of the CMA pathway (Cuervo and Dice, 2000*c*). Lamp2a is the only isoform of the *Lamp2* gene, which also includes Lamp2b and Lamp2c, with a role in CMA (Martinez-Vicente and Cuervo, 2007). Recently, it was shown that maintaining Lamp2a levels in the murine liver throughout life restored CMA activity, which was accompanied by the decreased accumulation of damaged proteins and the improvement of cellular homeostasis and overall hepatic function (Zhang and Cuervo, 2008).

Taken together, these findings point to a distinct role of CMA in the turnover of α-synuclein and the mediation of its toxic effects. Therefore, we carried out the present study to address whether boosting the function of CMA by overexpressing Lamp2a might reverse human α-synuclein-related neurotoxicity by reducing the levels of aberrant (high-molecular weight, phosphorylated) α-synuclein, while at the same time also mitigating its inhibitory effects on CMA.

Materials and methods

Generation of Lamp2a stable cell lines and transfections

For the generation of stable SH-SY5Y Lamp2a expressing lines, we used a mouse Lamp2a- haemagglutinin tagged plasmid (kindly provided by Dr Ana Maria Cuervo, Albert Einstein College of Medicine, USA). Two cell lines with low and high Lamp2a expression, as well as a line expressing the empty vector, were selected for future experiments (Supplementary material). Stable lines were maintained in growth medium (RPMI, 10% FBS) supplemented with 100 μg/ml Zeocin (Invitrogen). SH-SY5Y cells were differentiated in 10 μM all-trans retinoic acid for 5 days (Sigma).

Primary neuronal cultures

Cultures of rat (embryonic Day 18) cortical neurons were prepared as described previously (Vogiatzi *et al.*, 2008). The cells were plated onto poly-D-lysine-coated dishes at a density of ~150 000–200 000 cells/cm^2 and maintained in Neurobasal® medium, with B27 supplement (Invitrogen), L-glutamine (0.5 mM), and penicillin/streptomycin (1%).

Intracellular protein degradation

Total protein degradation was measured by labelling the cells with ^{3}H-leucine (2 μCi/ml) (Leucine, L-3,4,5, NEN-Perkin Elmer Life Sciences) using pulse-chase experiments as described previously (Vogiatzi *et al.*, 2008) (Supplementary material). To discriminate the contribution of each type of autophagy to the degradation of the long-lived proteins, blockers of lysosomal proteolysis were used. In particular, the general lysosomal inhibitors bafilomycin (500 nM, SH-SY5Y cells) or NH_4Cl (20 mM, cortical neurons) were used to assess total lysosomal proteolysis in SH-SY5Y cells and cortical neuron cultures, respectively. We have found bafilomycin to be more potent and consistent in inhibiting lysosomal degradation compared with NH_4Cl in SH-SY5Y cells, whereas both pharmacological agents are equivalent in inhibiting total lysosomal degradation in cortical neuron cultures

(Vogiatzi *et al.*, 2008; Xilouri *et al.*, 2009), hence their differential use in the two cellular systems. The inhibitor of phosphatidylinositol-3-kinase (PI3K) 3-methyladenine (10 mM, 3-methyladenine) was used to separate the percentage of total lysosomal degradation (bafilomycin or NH_4Cl-sensitive) that occurs via macroautophagy (3-methyladenine-sensitive). The remaining lysosomal degradation (insensitive to 3-methyladenine) can be attributed to microautophagy and CMA. Because the contribution of microautophagy in total lysosomal proteolysis is considered minor and the chase is performed in reduced (0.5%) serum conditions where CMA is induced, the majority of the proteolysis calculated by subtracting the 3-methyladenine-sensitive from the bafilomycin-or NH_4Cl-sensitive proteolysis is considered relative CMA degradation (Kaushik and Cuervo, 2009). Moreover, we have shown that Lamp2a downregulation in neuronal systems results in a commensurate reduction of 3-methyladenine-insensitive degradation measured by this assay, indicating that the above represents mainly CMA-dependent degradation (Vogiatzi *et al.*, 2008). Total radioactivity incorporated in cellular proteins was measured in a liquid scintillation counter (Wallac T414, Perkin Elmer) in triplicate samples per condition.

Measurement of endogenous α-synuclein half-life

Endogenous α-synuclein half-life in control and Lamp2a-expressing lines was assessed with pulse-chase experiments using a ^{35}S-methionine/cysteine mixture (0.2 mCi/ml) (Express Labeling Mix; PerkinElmer Life Sciences) as described previously (Vogiatzi *et al.*, 2008) (Supplementary material).

Production of recombinant adenoviruses

The complementary DNAs encoding wild-type α-synuclein, Lamp2a-haemagglutinin, and EGFP were cloned into a modified version of the pENTR.GD entry vector and introduced into the pAd/PL-DEST Gateway vector (Invitrogen). Second generation E1, E3, and E2a deleted recombinant human serotype 5 adenoviruses were generated as described previously (He *et al.*, 1998; Xilouri *et al.*, 2012) (Supplementary material). For viral infections, the adenoviruses were added to 5 days retinoic acid-differentiated SH-SY5Y cells (multiplicity of infection 50) or to primary cortical cultures (multiplicity of infection 100/virus) at 5 days after plating, for the designated time-points.

Assessment of survival

At the indicated time points following viral infections, SH-SY5Y cells or primary neurons were lysed in a detergent-containing solution, which enables the quantification of viable cells by counting the number of intact nuclei in a haemocytometer (Rukenstein *et al.*, 1991; Farinelli *et al.*, 1998). For specificity experiments, 6-day-old differentiated control or Lamp2a-expressing SH-SY5Y cells were treated with the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+; 250 μM, 24 h) or the DNA damaging agent camptothecin (5 μM, 24 h). Cell counts were performed in triplicate and are reported as means ± SEM.

Production of recombinant adeno-associated viruses

Recombinant adeno-associated viruses (AAVs) expressing GFP, human α-synuclein and Lamp2a were generated as described previously (Grimm *et al.*, 1998; Zolotukhin *et al.*, 1999) (Supplementary material).

The final titre of the used recombinant AAVs was 8E14 genome copies/ml, 9.4E14 genome copies/ml, and 3.2E14 genome copies/ml for recombinant AAV6 expressing Lamp2a, wild-type α-synuclein, and GFP, respectively.

Animals

Eight-week-old female Sprague-Dawley rats (200–225 g) were purchased from Charles River and 2–3 animals were housed per cage with free access to food and water under a 12 h light/dark cycle. All experimental procedures performed were approved by the Ethical Committee for Use of Laboratory Animals in the Lund-Malmö region.

Surgical procedure

All surgical procedures were performed under general anaesthesia using ~6 ml/kg of a 20:1 mixture of fentanyl and Dormitor® (injected intraperitoneally). After placing the animal into a stereotaxic frame (Stoelting), 2 μl of recombinant AAV6 solution was injected unilaterally into the substantia nigra using the following coordinates: −4.8 mm anteroposterior, −2.0 mm mediolateral from the bregma, and −7.2 mm dorsoventral from the dura, according to the rat stereotaxic atlas (Paxinos and Watson, 1998). The tooth bar was adjusted to −2.3 mm. Injection was performed using a pulled glass capillary (diameter of ~60–80 μm) attached to a Hamilton syringe with a 22 s gauge needle. After delivery of the viral vector using an injection rate of 0.1 μl/15 s the capillary was held in place for 5 min, retracted 0.1 μm, and, after 1 min, was slowly withdrawn from the brain.

Western blotting

SH-SY5Y cells and primary neurons were washed twice in cold PBS and then harvested in lysis buffer (150 mM NaCl, 50 mM Tris pH 7.6, 1% Triton™ X-100, 2 mM EDTA) with protease inhibitors, as described previously (Vogiatzi *et al.*, 2008). For the biochemical analysis of the ventral midbrain and striatal tissues, the animals were decapitated at 8 weeks following injection; the brains were harvested, dissected on ice to obtain the region of interest, and frozen immediately. All animals were processed in a similar manner. Tissue was stored at −80°C until further use. The ventral midbrain encompassing the substantia nigra and the striata from the injected and uninjected hemispheres were rapidly dissected and homogenized in Triton extraction buffer (150 mM NaCl, 50 mM Tris pH 7.6, 1% Triton™ X-100, 2 mM EDTA) containing phosphatase inhibitors. The lysate was sonicated and then centrifuged (50 000 *g* for 60 min) to collect the supernatant ('Triton-X soluble fraction'). The pellet was incubated with lysis buffer supplemented with 2% SDS, heated at 95°C for 30 min, and centrifuged under the same conditions. The resulting supernatant represents the 'Triton-X insoluble fraction' (SDS soluble). The intensity of the immunoreactive bands was estimated by densitometric quantification using the Gel Analyzer v1.0 software (Supplementary material).

High-performance liquid chromatography

Analysis of striatal dopamine content was performed as described previously (Pitychoutis *et al.*, 2009; Xilouri *et al.*, 2012), with minor modifications (Ulusoy *et al.*, 2009). Briefly, the two striata (from contra- and ipsilateral hemispheres) were individually sonicated in ice-cold Tris-acetate-EDTA buffer (20 mM, pH 6.1). Perchloric acid (70%) was directly added to the homogenate in order to obtain a final concentration

of 0.4 M. After appropriate mixing, samples were incubated for 20 min on ice and were then centrifuged at 10 000 *g* for 20 min at 4°C. The supernatant was then collected and stored at −80°C until analysis. Samples were diluted 1:3 with double-distilled H_2O before being injected to a BAS-LC4B HPLC system with an amperometric detector (Supplementary material). Results are expressed as % dopamine of contralateral side. The limit of detection was 1 pg/27 µl (injection volume).

Immunocytochemistry

SH-SY5Y cells or cortical neurons grown on 24-well plates were fixed in freshly prepared 3.7% formaldehyde for 25 min. Blocking was with 10% normal goat serum and 0.4% Triton™ X-100 for 1 h at room temperature. Mouse haemagglutinin-tag (6E2) (1:400; Cell Signaling, 2367) antibody was applied overnight at 4°C, followed by a fluorescent secondary antibody (mouse Cy3, 1:250; Jackson ImmunoResearch) for 1 h at room temperature. The fluorescent marker Hoechst 33258 (1 µM; Sigma) was used to assess cell nuclei.

Immunohistochemistry

Animals were perfused intracardially through the ascending aorta with physiological saline under pentobarbital anaesthesia, followed by ice-cold 4% paraformaldehyde. The brains were post-fixed for 1 h in the same preparation of paraformaldehyde and then transferred to 25% sucrose until sectioning. The brains were sectioned through the coronal plane at 35 µm increments, and every section throughout the striatum and the substantia nigra were collected. Immunohistochemical staining was carried out in free-floating sections as described previously (Xilouri *et al.*, 2012) (Supplementary material).

Stereology and striatal tyrosine hydroxylase immunoreactive density

Number of tyrosine hydroxylase (TH)-positive nigral neurons was estimated by an unbiased stereological quantification method using the optical fractionator principle embedded in the Stereo Investigator v10.0 software (MBF Bioscience), a motorized stage (MAC5000, MBF Bioscience) and a top-mounted camera (QImaging, MBF Bioscience) with the light microscope (Leica, DMRA2). Every sixth section throughout the whole rostro-caudal axis of the substantia nigra was included, region of interest was outlined with a ×4 objective and counting was performed using a ×60 oil objective. Random start and systematic sampling was applied. Counting parameters were adjusted to achieve at least 100 counts per hemisphere. All quantifications and analysis were performed by a single blinded investigator. A coefficient of error (Gundersen) of <0.1 was accepted. Data are means from all brains and expressed as % of contralateral side.

Striatal TH-density was quantified using four coronal sections per brain, covering the whole rostro-caudal axis of the striatum. Densitometric analysis was performed on images taken of the sections, and expressed as % of contralateral.

Behavioural testing

Seven weeks post-viral injections, the animals were injected with 2.5 mg/kg D-amphetamine intraperitoneally or 0.25 mg/kg apomorphine subcutaneously and placed into an acrylic glass bowl with a harness fitted behind the forepaws. The harness was connected to a computerized rotation counter that registers every 90° full body turn (AccuScan Instruments Inc.). The measurement time was 90 min or 40 min for D-amphetamine or apomorphine, respectively. The data are expressed in mean net turns per minute ipsilateral to the injection site.

Statistical analysis

The data are shown as the mean ± SEM. Unless stated otherwise, statistical analysis was carried out with GraphPad Prism 5 using one-way or two-way ANOVA followed by Bonferroni's multiple comparisons post-test, as indicated. Differences were considered significant for $P < 0.05$.

Results

Generation and characterization of human SH-SY5Y cell lines overexpressing Lamp2a

In order to investigate the effects of CMA induction on α-synuclein levels and related toxicity, we first generated human SH-SY5Y neuroblastoma cell lines stably expressing the lysosomal receptor Lamp2a. We selected two clones expressing low (low Lamp2a line) and high (high Lamp2a line) levels of haemagglutinin-tagged Lamp2a, and a control line (empty vector) generated by transfection with the empty vector. Stable expression of Lamp2a receptor was detected in both Lamp2a-expressing lines as assessed by immunofluorescence microscopy and western immunoblotting (Fig. 1A and B). Quantification of Lamp2a protein levels revealed that Lamp2a was expressed 1.2 and 4.5-fold above the endogenous levels in the low and high Lamp2a lines, respectively (Fig. 1C). To test the functionality of the overexpressed Lamp2a, we assessed relative CMA and total lysosomal proteolysis using a long-lived protein degradation assay, where the percentage of total lysosomal proteolysis (bafilomycin-sensitive), the macroautophagic degradation (3-methyladenine-sensitive) and the non-macroautophagic degradation (mainly CMA) can be assessed, as described previously (Xilouri *et al.*, 2008). Indeed, we observed that CMA activity, estimated as (bafilomycin − 3-methyladenine)-sensitive degradation, was increased in the Lamp2a-expressing lines compared with the control line (empty vector), in a manner that was dependent upon Lamp2a expression levels (Fig. 1D).

There was no significant difference in the steady-state levels of endogenous α-synuclein between the three cell lines in full serum (10% foetal bovine serum) conditions (Fig. 1E) or in reduced serum (0.5 %) conditions (data not shown). As steady-state α-synuclein levels did not change, even though increased 3-methyladenine-insensitive proteolysis was observed, we then investigated whether the turnover of endogenous α-synuclein was different between the cell lines using pulse-chase experiments. For the chase, we used full serum (10%) or reduced serum (0.5%, induction of CMA) conditions in order to detect potential changes upon maximum CMA activation. In both conditions, the half-life of endogenous α-synuclein was significantly reduced in the high Lamp2a-expressing line (Fig. 1F and G). Real-

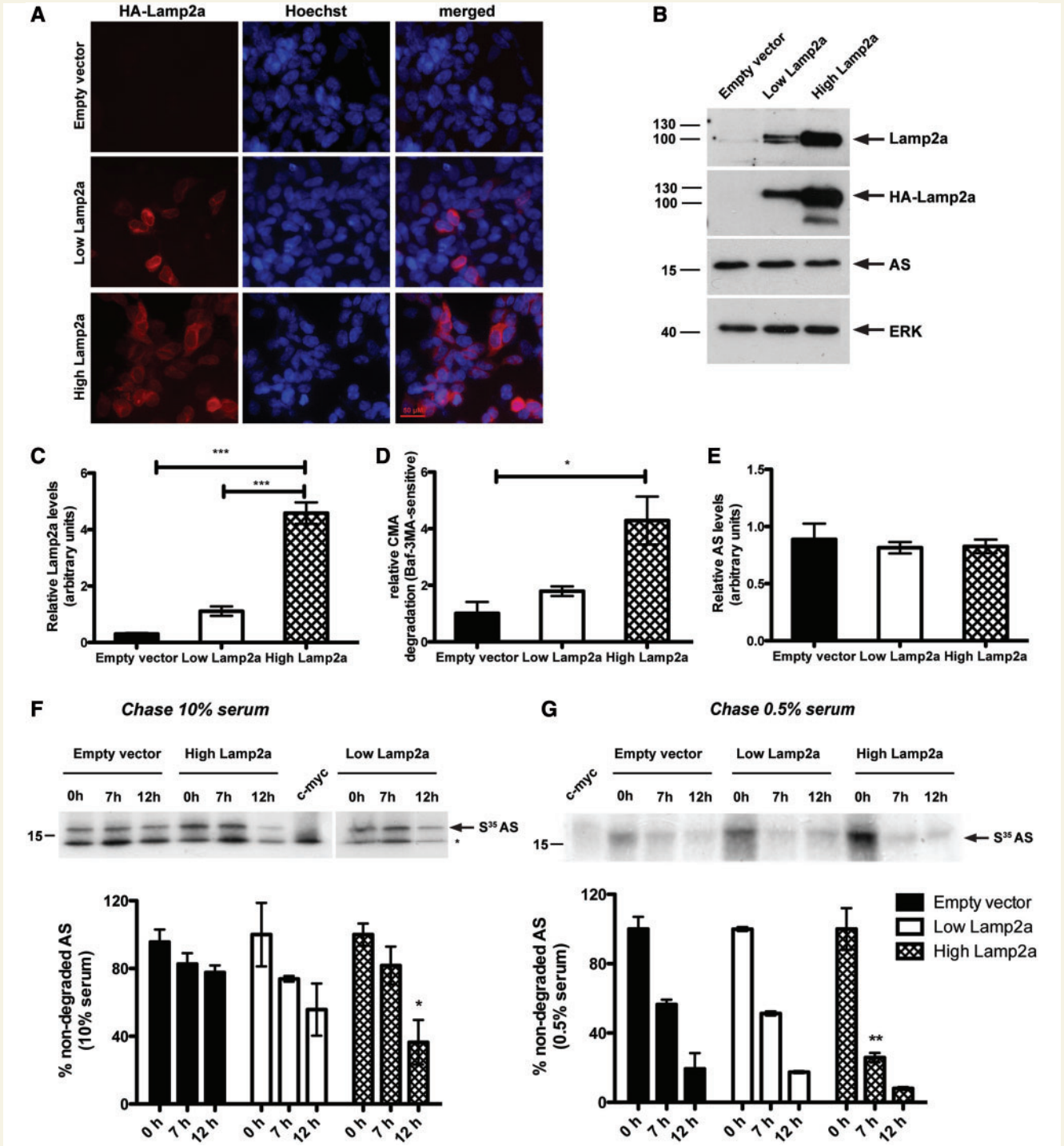

Figure 1 Human SH-SY5Y cell lines stably expressing Lamp2a display increased CMA activity, which is accompanied by the increased turnover of endogenous α-synuclein. Scale bar = 50 μm. (**A**) Representative immunostaining for haemagglutinin (HA) (Lamp2a-tag) in the empty vector and the Lamp2a (low and high)-expressing lines. Scale bar = 50 μm. (**B**) Western immunoblot for Lamp2a, HA-Lamp2a, α-synuclein (AS), and ERK (loading control) levels in all lines. (**C**) Quantification of relative Lamp2a protein levels in all lines (***$P < 0.001$; $n = 3$). (**D**) Relative non-macroautophagic [bafilomycin-3-methyladenine (Baf-3MA)-sensitive] proteolysis in control and Lamp2a-expressing lines (*$P < 0.05$; $n = 3$ with triplicate samples within each experiment). (**E**) Quantification of relative α-synuclein steady-state levels using the C20 α-synuclein-specific antibody (ab). (**F** and **G**) *Top*: Representative pulse-chase experiments in control and Lamp2a-expressing lines in 10% (**F**) or 0.5% (**G**) serum. α-synuclein was immunoprecipitated (IP) from hot lysates using the syn-1 antibody, and its levels were assessed by autoradiography. The band corresponding to α-synuclein is depicted by the arrow, while the asterisk indicates an irrelevant band (c-myc IP was used as negative control). *Bottom*: Quantification of α-synuclein turnover. Data are presented as the relative optical density (OD) values of each time point relative to time point 0 (*$P < 0.05$; **$P < 0.01$, comparing between empty vector and Lamp2a-expressing lines, two-way ANOVA; $n = 3$).

time PCR experiments revealed similar α-synuclein messenger RNA levels between the lines (Supplementary Fig. 1), indicating that differential messenger RNA levels could not account for the discrepancy between the similar steady-state protein levels of α-synuclein and the reduced half-life with Lamp2a overexpression. It is possible instead that the difference in protein synthesis may be responsible for this effect.

Differentiated SH-SY5Y lines overexpressing Lamp2a are selectively protected against adenoviral-mediated wild-type α-synuclein-induced neurotoxicity

To assess whether the upregulation of CMA could diminish α-synuclein-related toxicity, we transduced control or Lamp2a-expressing lines with adenoviruses expressing wild-type α-synuclein or EGFP (control virus). These experiments were performed in differentiated cells (10 μM retinoic acid, 5 days), because overexpression of α-synuclein with adenoviruses was not toxic to proliferating cells (data not shown). The overexpression of wild-type α-synuclein (multiplicity of infection = 50, 72 h) reduced the survival of differentiated control (empty vector) cells, whereas this was restored to basal levels in the Lamp2a-expressing lines, in a manner that was dependent on Lamp2a expression levels. This neuroprotection was selective since the Lamp2a-expressing cells were not protected against other toxic insults such as MPP^+ and camptothecin (Fig. 2A). Detection of total α-synuclein levels (endogenous and overexpressed) by immunoblotting did not show any significant difference in α-synuclein levels between control or Lamp2a-expressing lines, although the high Lamp2a line displayed slightly faster wild-type α-synuclein turnover at a later time point (Fig. 2B). We cannot exclude the possibility that the constant expression of the transgene with the adenoviruses may have masked a potential effect on the degradation of the protein. Assessment of lysosomal function at 72 h post-transduction showed that the high Lamp2a-expressing cells retained statistically significant higher rates of relative CMA-dependent proteolysis, upon EGFP or wild-type α-synuclein treatment (Fig. 2C), compared with the control line, whereas no difference was detected in total (bafilomycin-sensitive) lysosomal proteolysis (Fig. 2D). The low Lamp2a-expressing line showed intermediate values of CMA-dependent degradation.

Transduction of empty vector cells with wild-type α-synuclein adenovirus resulted in decreased (though not statistically significant) CMA-sensitive proteolysis compared with transduction with EGFP (Fig. 2C). This does represent a definite trend, which is reversed in the Lamp2a-overexpressing lines. Definitive evidence for CMA dysfunction is difficult to achieve in this system, owing to the low baseline levels of CMA activity. Given the fact that induction of CMA activity in the presence of α-synuclein transduction is definitively demonstrated with high Lamp2a expression, we conclude that, in this system, Lamp2a ameliorates wild-type α-synuclein-induced toxicity likely through maintaining high CMA activity.

Lamp2a overexpression in rat primary cortical cultures induces relative chaperone-mediated autophagy-dependent proteolysis, reduces total α-synuclein protein burden and ameliorates wild-type α-synuclein-induced neurotoxicity

Even though the aforementioned Lamp2a-expressing lines represent a valuable tool to study the effects of CMA induction in a human dopaminergic cell line setting, there are some limitations that must be taken into consideration, such as the heterogeneity of the cells expressing Lamp2a, especially in the low Lamp2a line. Therefore, the actual effects of Lamp2a overexpression might be masked using experimental approaches based on assessment of the whole cell population. Furthermore, detrimental effects on CMA activity with α-synuclein overexpression were not conclusively demonstrated in this system, and we could not be completely certain that the observed effects in the Lamp2a-expressing lines did not represent clonal variability. In order to overcome these issues, and to confirm these results in another neuronal cell culture system, we have used primary cortical neuronal cultures, where Lamp2a could be homogenously overexpressed through adenoviruses. These cultures represent a rich pure source of homogeneous post-mitotic neurons that are sensitive to α-synuclein accumulation in patients with Parkinson's disease, albeit at later stages of the disease (Braak *et al.*, 2003). Previously, we showed that downregulation of Lamp2a in primary rat cortical neurons increases endogenous α-synuclein levels (Vogiatzi *et al.*, 2008), whereas adenoviral-expressed wild-type α-synuclein is toxic in this system (Xilouri *et al.*, 2009). To investigate whether CMA targeting may have beneficial effects on this model of α-synuclein-mediated neurotoxicity, we generated recombinant adenoviruses overexpressing mouse Lamp2a (haemagglutinin tagged). Transduction of rat primary neurons (embryonic Day 18, E18) at *in vitro* Day 5 with the Lamp2a-expressing adenovirus resulted in the robust overexpression of Lamp2a as detected by immunohistochemistry (note the characteristic punctuate staining of Lamp2a in the cell body and neuritic processes in Fig. 3A) and by western immunoblotting for haemagglutinin tag at 72 h post-transduction (Fig. 3B). Lamp2a overexpression in cortical neurons was followed by a 3-fold induction of the 3-methyladenine-insensitive proteolysis, indicative of increased CMA activity (Fig. 3C). Interestingly, this induction was followed by a slight decrease in macroautophagic (3-methyladenine-sensitive) degradation (Fig. 3C), suggesting the presence of a compensatory mechanism between these two autophagic mechanisms.

We went on to investigate whether CMA targeting could mitigate wild-type α-synuclein-induced neurotoxicity in this system. To this end, we transduced 5-day-old cortical neurons with adenoviruses in the following pairs: wild-type α-synuclein + Lamp2a, wild-type α-synuclein + EGFP, or EGFP alone with the same total viral load (multiplicity of infection = 200). The effects on α-synuclein protein levels, lysosomal function, and neuronal survival were

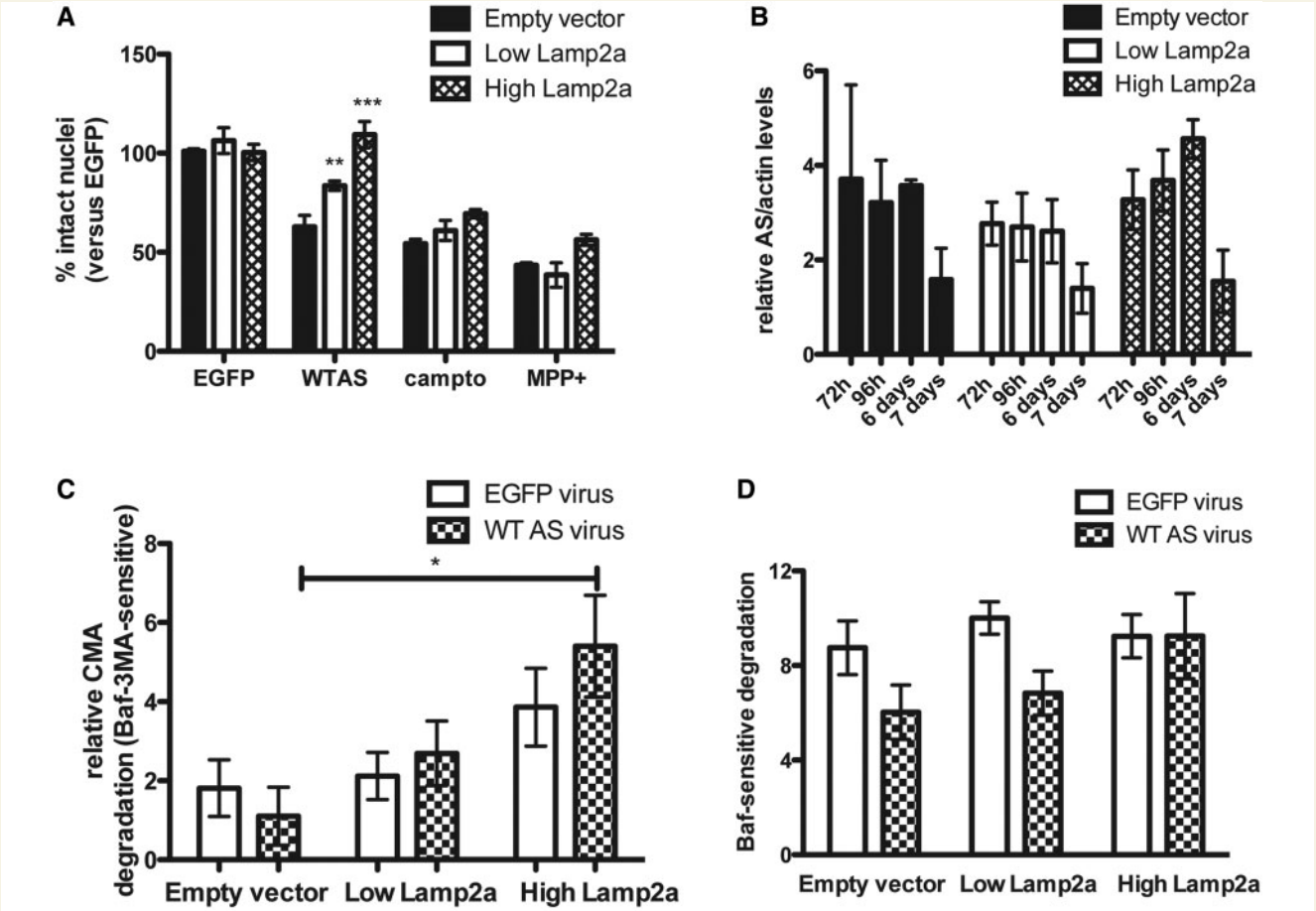

Figure 2 Lamp2a overexpression selectively protects differentiated SH-SY5Y against adenoviral (AV) mediated wild-type α-synuclein (WT AS)-induced neurotoxicity. (**A**) Survival of differentiated control or Lamp2a-expressing lines transduced with EGFP or wild-type α-synuclein adenovirus (multiplicity of infection = 50, 72 h), or treated with MPP^+ (250 μm, 24 h) or camptothecin (5 μm, 24 h) was assessed by counting the number of intact nuclei (*$P < 0.05$; **$P < 0.01$; comparing between empty vector and Lamp2a-expressing lines upon wild-type α-synuclein treatment, two-way ANOVA; $n = 4$, with triplicate samples within each experiment). (**B**) Quantification of relative wild-type α-synuclein levels (C20 antibody) on western immunoblots, with β-actin as the loading control, at successive time points after viral transduction with wild-type α-synuclein adenovirus ($n = 3$). (**C** and **D**) Relative CMA-dependent (bafilomycin–3-methyladenine-sensitive) and total lysosomal-dependent (bafilomycin-sensitive) degradation were assessed after viral transduction with EGFP or wild-type α-synuclein adenoviruses (multiplicity of infection = 50, 72 h) (*$P < 0.05$; comparing between empty vector and high Lamp2a lines transduced with wild-type α-synuclein adenovirus; $n = 3$ with triplicate samples within each experiment).

estimated at 96 h (Fig. 3D–F) and 7 days (Fig. 3G–I) post-transduction. Double immunostaining for α-synuclein and Lamp2a-haemagglutinin revealed that the majority of the transduced cells expressed both transgenes (data not shown). At 96 h post-transduction, Lamp2a co-expression did not change total α-synuclein levels (endogenous and transduced) (Fig. 3D), but led to significant amelioration of CMA-dependent proteolysis, which was significantly impaired by α-synuclein overexpression (Fig. 3E). By 7 days of transduction, Lamp2a co-expression led to a significant decrease of total α-synuclein levels (Fig. 3G), while still providing significantly enhanced CMA-dependent degradation (Fig. 3H). At both time points, Lamp2a co-expression conferred significant protection against α-synuclein-induced death (Fig. 3F and I). Finally, assessment of autophagic flux in these cultures by measuring the protein levels of LC3-II and p62 did not reveal any significant difference between the treatments, indicating that macroautophagy was not significantly affected (data not shown).

Efficient delivery of Lamp2a through a recombinant adeno-associated virus to the rat substantia nigra

Our ultimate goal was to elucidate whether the modulation CMA by way of Lamp2a overexpression could represent a viable therapeutic strategy *in vivo*. To accomplish this, we utilized a well-established model of *in vivo* synucleinopathy entailing the direct stereotaxic delivery of wild-type α-synuclein-overexpressing recombinant AAV to the rat substantia nigra pars compacta (Kirik *et al.*, 2002). Initial injections using three different titres of

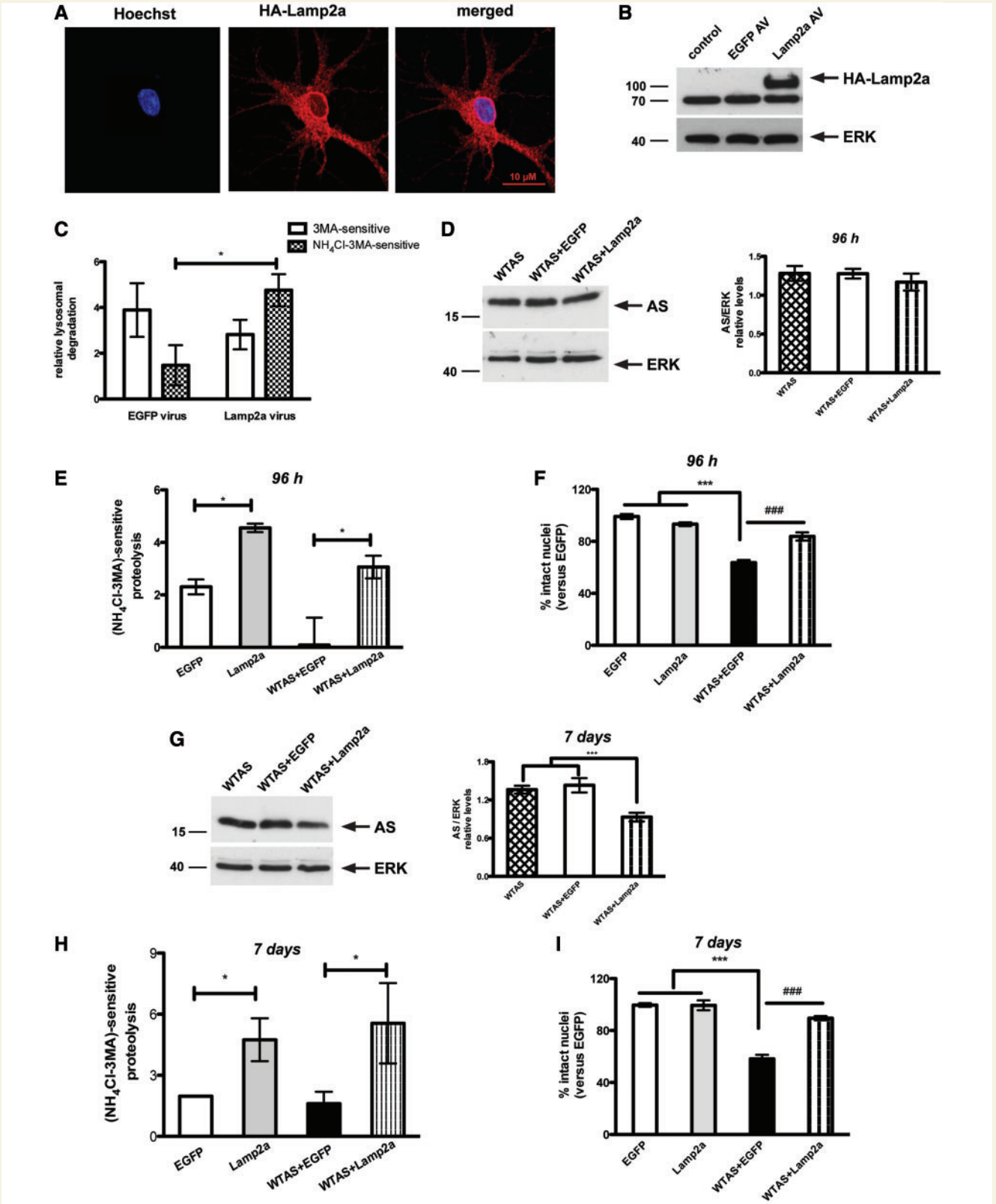

Figure 3 Lamp2a overexpression in primary neurons reverses wild-type α-synuclein (WTAS)-mediated neurotoxicity, mainly through restoration of CMA activity and, at later time-points, through the reduction of α-synuclein protein burden. (**A**) Representative immunostaining for haemagglutinin (HA) (Lamp2a adenovirus, multiplicity of infection = 100) at 96 h post-transduction. Scale bar = 10 μm. (**B**) Representative western immunoblot for haemagglutinin-Lamp2a and ERK (loading control) levels in control (untransduced) and Lamp2a adenovirus- or EGFP adenovirus-transduced cortical cultures at 96 h post-transduction. (**C**) Relative macroautophagy-dependent

(continued)

recombinant AAV-Lamp2a (ranging from 1.2×10^{13} to 2.9×10^{14} genome copies/ml) or control GFP recombinant AAV (2×10^{14} genome copies/ml) were performed to test the efficacy of transduction, the functionality of the Lamp2a transgene, and the impact on the integrity of the dopaminergic system. Eight weeks following injections, nigral sections were double-stained with antibodies against TH in combination with haemagglutinin (Lamp2a-tag) or GFP, where relevant. We confirmed that all recombinant AAV-Lamp2a viral titres were highly expressed in the nigral dopaminergic (TH+) neurons (> 90%) throughout the whole nigral rostrocaudal axis (Fig. 4A illustrates selected levels), similar to the GFP-expressing recombinant AAV. Compared to the injected side, only weak expression of endogenous Lamp2a could be discerned in the contralateral non-treated substantia nigra, with no immunoreactivity to haemagglutinin-tagged Lamp2a (Fig. 4B). The overexpressed Lamp2a was targeted to the lysosomes of the transduced nigral neurons, as overexpressed Lamp2a co-localized strongly in discrete perinuclear puncta with the lysosomal marker Lamp1 (Fig. 4C). Comparing transduced versus non-transduced neurons in the ipsi- and contralateral side of the Lamp2a injected animals, we observed a discernible mobilization of overexpressed Lamp2a-enriched lysosomes to the perinuclear region of substantia nigra neurons (Supplementary Fig. 2A), which is an indirect indicator of increased CMA activity (Kaushik and Cuervo, 2009). Western blot analysis of the various subcellular (cytosolic, mitochondrial and lysosomal) fractions obtained through a discontinuous Nycodenz® gradient method, as described previously (Kaushik and Cuervo, 2009), revealed that the overexpressed Lamp2a was enriched in the lysosomal fractions that were also positive for the activity of the well-characterized lysosomal enzyme β-hexosaminidase (data not shown) while, at the same time, endogenous α-synuclein levels were found decreased in the Lamp2a transduced animals, in a manner that was dependent on Lamp2a expression levels (Supplementary Fig. 2B and C). Due to the limited starting material (half midbrain per condition), the resulting lysosomal fractions were not sufficient to perform *in vitro* CMA activity assays. We subsequently assessed the integrity of the dopaminergic system under these conditions. High performance liquid chromatography measurements did not reveal any significant difference in dopamine content between the contra- and ipsilateral hemispheres in all groups (Fig. 4D). In a similar fashion, no significant difference in the number of TH+ neurons was observed between the contra- and ipsilateral sides, even for a high titre of Lamp2a-expressing recombinant AAV (Fig. 4E).

Complete restoration of human α-synuclein-mediated nigrostriatal degeneration through nigral Lamp2a overexpression

In a second set of injections, we investigated the effect of Lamp2a overexpression on wild-type α-synuclein-induced neurotoxicity in the rat substantia nigra. To this end, we performed injections of recombinant AAVs in three groups: GFP + Lamp2a, GFP + α-synuclein, and α-synuclein + Lamp2a, with the same total viral load (8×10^{11} genome copies). Eight weeks following vector delivery, the majority of transduced neurons co-expressed both transgenes in all groups (Fig. 5A), which was estimated at ~90% upon quantification of double-positive cells (data not shown). Notably, in the GFP + α-synuclein group, strong diffuse cytoplasmic α-synuclein immunoreactivity was observed (Fig. 5A), whereas in the α-synuclein + Lamp2a group, α-synuclein immunoreactivity was restricted to a peripheral thin ring-shaped pattern (Fig. 5A), suggesting that the co-expression of Lamp2a altered the distribution and, possibly, the expression levels of α-synuclein.

The potential neuroprotective effects of Lamp2a overexpression on wild-type α-synuclein-induced dopaminergic degeneration was estimated by stereological cell counting of TH+ neurons in the substantia nigra. Measurements between the ipsilateral side relative to the contralateral side revealed that the GFP + α-synuclein animals displayed a $49.8 \pm 6.8\%$ loss of dopaminergic neurons compared with the $20 \pm 5.1\%$ loss of the control GFP + Lamp2a animals (Fig. 5B–D). The 20% loss of TH+ neurons observed in the ipsilateral side of the control group was due to the relative toxicity of GFP protein overexpression and/or the surgical procedure. Notably, the dopaminergic cell loss caused by α-synuclein overexpression was completely restored to control levels ($15.8 \pm 10.4\%$) in the α-synuclein + Lamp2a-injected animals (Fig. 5B–D). The Lamp2a-neuroprotective effect was not only restricted to nigral cell bodies but was also observed in the dopaminergic terminals, as estimated by measurements of the immunoreactive density of striatal TH+ fibres (Fig. 5E and F) and of striatal dopamine levels by HPLC (Fig. 5G). Additionally, western blot analysis of the striatal samples derived from the GFP + α-synuclein group showed that TH protein levels were reduced by ~20% and total α-synuclein levels were increased by ~1.5-fold, while both phenomena were restored to control levels in the α-synuclein + Lamp2a group (Supplementary Fig. 3). In all groups, we also employed a motor behaviour test, i.e.

Figure 3 Continued
(3-methyladenine-sensitive) and non-macroautophagic (NH_4Cl–3-methyladenine-sensitive) proteolysis at 96 h after transduction with Lamp2a or EGFP adenovirus (*$P < 0.05$; $n = 4$ with triplicate samples within each experiment). (**D** and **G**) A representative western blot for α-synuclein (C20 antibody) at 96 h (**D**) or 7 days (**G**) post-transduction is shown in the *left* panel and quantification of α-synuclein levels is shown on the *right*. An ERK antibody was used as a loading control (***$P < 0.001$). (**E** and **H**) Relative non-macroautophagic (NH_4Cl–3-methyladenine-sensitive) proteolysis of cortical neurons assessed at 96 h (**E**) and at 7 days (**H**) post-transduction (*$P < 0.05$). (**F** and **I**) Survival of cortical neurons expressed as percentage of control EGFP adenovirus-infected cultures at 96 h (**F**) or 7 days (**I**) post-transduction (***$P < 0.001$, comparing between EGFP or Lamp2a and wild-type α-synuclein + EGFP-transduced cultures; $^{###}P < 0.001$, comparing between wild-type α-synuclein + EGFP and wild-type α-synuclein + Lamp2a-transduced cultures; $n = 3$ with triplicate samples within each experiment).

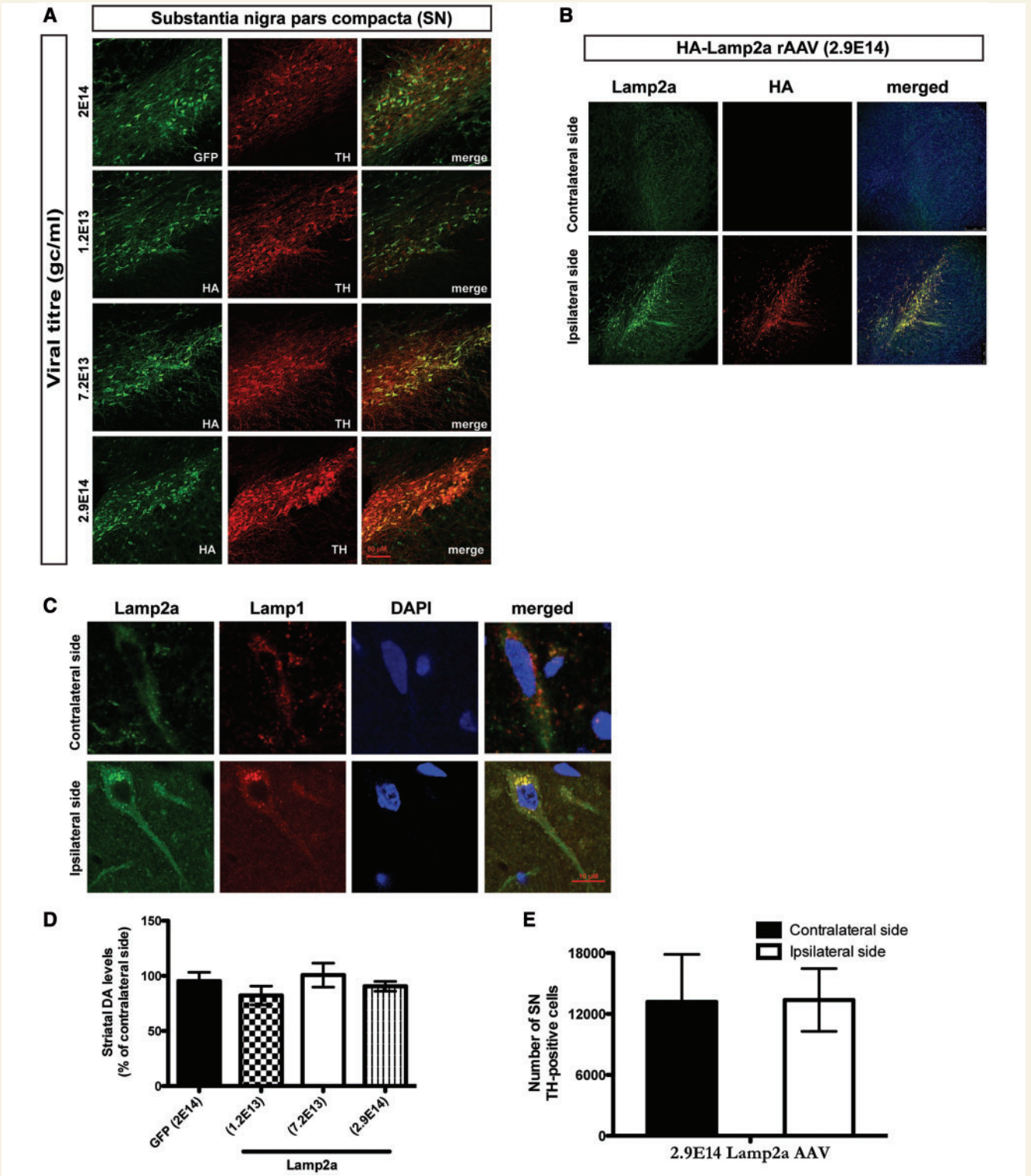

Figure 4 Recombinant AAV-mediated delivery of Lamp2a in the rat substantia nigra results in the efficient transduction of dopaminergic neurons at 8 weeks later without aberrant effects on the dopaminergic system. (**A**) Representative immunofluorescent images showing the expression of recombinant AAV-Lamp2a and control recombinant AAV-GFP (2E14 genome copies/ml) (green) in TH^+ dopaminergic neurons (red). Scale bar = 50 μm. (**B**) Representative immunohistochemical fluorescent images with Lamp2a and haemagglutinin antibodies showing the induction of Lamp2a expression in the ipsilateral side following injection with recombinant AAV-Lamp2a (7.2E13). Scale bar = 250 μm. (**C**) Representative immunofluorescence images showing the punctated co-localization of Lamp2a (green, endogenous and overexpressed) with the lysosomal marker Lamp1 (red). Scale bar = 10 μm. (**D**) High-performance liquid chromatography analysis of striatal dopamine (DA) levels expressed as the mean % of the contralateral side (n = 5/group). (**E**) Stereological estimates of the density of TH + neurons in the substantia nigra of mid-titre (7.2E13) recombinant (r) AAV-Lamp2a-injected animals (n = 3/hemisphere).

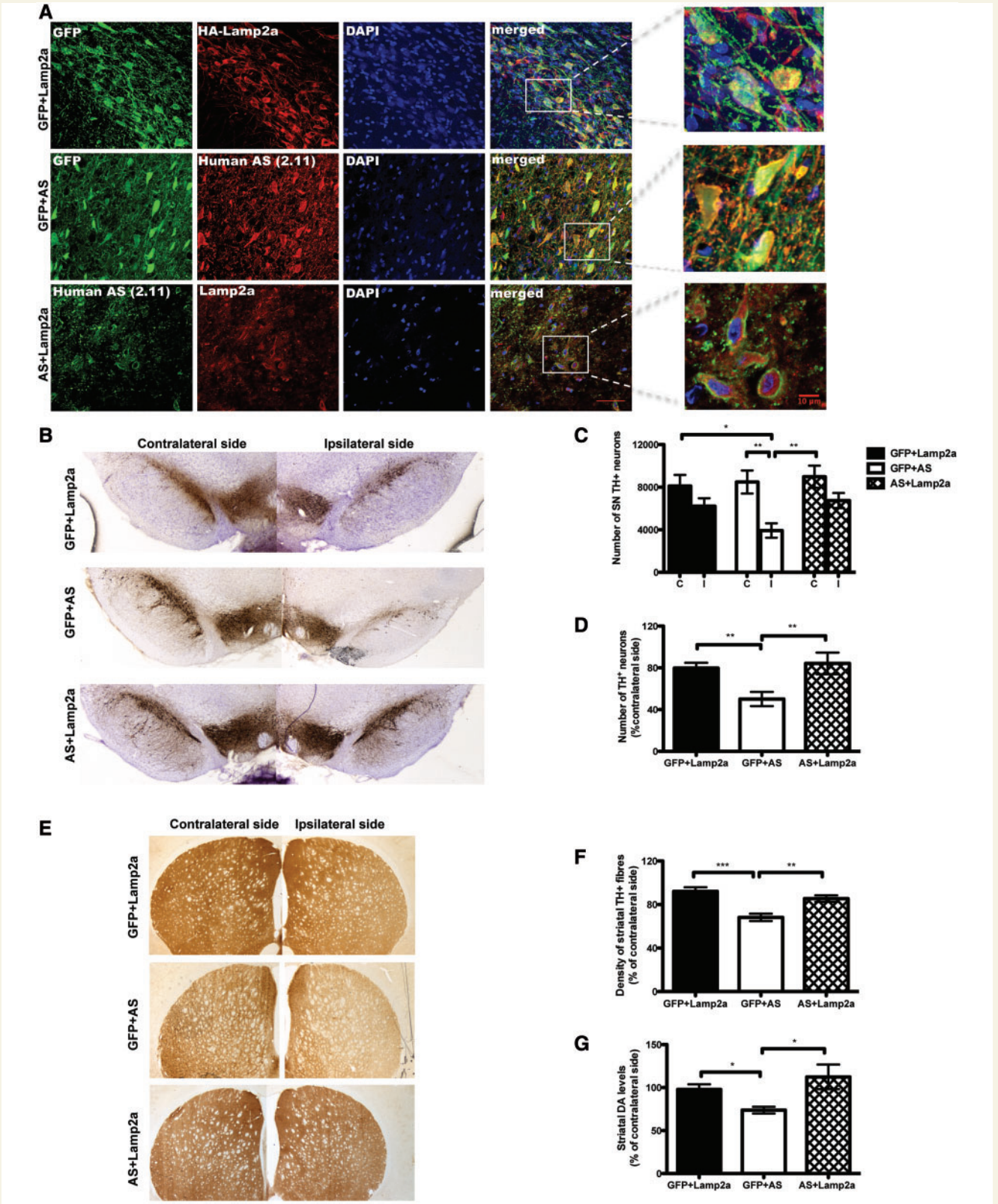

Figure 5 Complete restoration of human α-synuclein (AS)-mediated nigrostriatal degeneration through Lamp2a overexpression in the rat substantia nigra. (**A**) Representative immunofluorescent images showing the co-localization of GFP (green) and Lamp2a [haemagglutinin (HA), red, *top* row] in the GFP + Lamp2a treated animals, GFP (green) and human α-synuclein (clone 2.11, red, *middle* row) in the GFP + α-synuclein animals, and human α-synuclein (2.11, green, *bottom* row) and Lamp2a (red) in the α-synuclein + Lamp2a injected animals at 8 weeks post-injection. Scale bar = 50 μm. High-power merged images are shown on the right. Scale bar = 10 μm.

(continued)

the measurement of amphetamine-induced rotations. Likely due to the partial nature of the lesions, the GFP + α-synuclein-injected rats showed only a small, non-significant increase of rotation behaviour compared with the GFP + Lamp2a controls. The α-synuclein + Lamp2a-injected rats had a similar profile to the controls, suggesting a reversal of this slight behavioural abnormality (Supplementary Fig. 4).

Targeting of the chaperone-mediated autophagy pathway through Lamp2a upregulation decreases total α-synuclein levels and diminishes the generation of Parkinson's disease-linked aberrant α-synuclein species

To assess whether *in vivo* Lamp2a overexpression, in addition to protecting against α-synuclein-induced dopaminergic neurodegeneration, may also limit the total α-synuclein burden, we biochemically assessed the generated α-synuclein species in ventral midbrain extracts from animals injected with GFP + α-synuclein and α-synuclein + Lamp2a. To this end, we performed sequential fractionation of ventral midbrain tissue encompassing the substantia nigra, initially in a 1% Triton^{TM} extraction buffer (Triton-X soluble fraction) and subsequently in a 2% SDS extraction buffer (SDS soluble fraction). Using this *in vivo* model of synucleinopathy, we detected a 4-fold increase in the levels of monomeric α-synuclein in the injected midbrain (data not shown), as measured by western blot analysis using the C20 α-synuclein antibody that recognizes both endogenous and overexpressed protein. Moreover, phosphorylated (S129, p-α-synuclein) and human-specific (stained with LB509 antibody) α-synuclein species were detected in the Triton-X soluble fractions derived from the ipsilateral side of both groups, while such species were absent in the contralateral side (Fig. 6A). Only monomeric α-synuclein was detected in the Triton-X soluble fractions derived from both groups (Fig. 6B). Importantly, Lamp2a overexpression reduced relative p-α-synuclein and monomeric human-specific α-synuclein levels (Fig. 6C). In the Triton-X-insoluble but SDS soluble fractions, monomeric and higher molecular weight α-synuclein species accumulated on the side of the injection, as detected with two different α-synuclein-specific antibodies (monoclonal syn-1 and polyclonal C20) (Fig. 6D). Strikingly, the levels of these aberrant monomeric and high-molecular-weight α-synuclein species were significantly reduced when Lamp2a was co-expressed with α-synuclein (Fig. 6E and F). Assessment by western immunoblotting of potential alterations in the levels of macroautophagy markers, e.g. LC3-II and p62, showed no significant change between the groups (data not shown).

Discussion

A major theory regarding the pathogenesis of Parkinson's disease and other synucleinopathies posits that the culprit is an excess burden of α-synuclein, especially aberrant oligomeric and aggregated protein conformations. We and others have shown that wild-type α-synuclein is degraded through the CMA pathway in neuronal cells, whereas the Parkinson's disease-linked mutant α-synuclein forms resist this degradation and may act as inhibitors of CMA. We hypothesized that the induction of CMA activity may halt α-synuclein-induced neurodegeneration through two different mechanisms: limiting α-synuclein protein burden and mitigating its detrimental effects on lysosomal function. To investigate this hypothesis, we have taken the approach of inducing CMA activity through Lamp2a overexpression.

The selection of Lamp2a as a potential target was based on reports showing that Lamp2a levels correlate directly with the rate of CMA activity under various physiological and pathological conditions (Cuervo and Dice, 2000*a*, *b*; Zhang and Cuervo, 2008). In relation to α-synuclein, we have shown that silencing of Lamp2a increased total α-synuclein levels as well as high-molecular-weight Triton-X-soluble and insoluble species in various neuronal systems (Vogiatzi *et al.*, 2008). Additionally, another Parkinson's disease-related pathogenic protein, the I93M mutant of ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase) (UCHL1), induces a CMA inhibition-associated increase in the amount of α-synuclein through its aberrant interaction with Lamp2a (Kabuta *et al.*, 2008). Furthermore, Alvarez-Erviti *et al.* (2010) reported decreased Lamp2a and Hsc70 levels in α-synuclein inclusion-forming regions of the substantia nigra and amygdala of patients with Parkinson's disease compared with control subjects and patients with Alzheimer's disease, suggesting that this deterioration of CMA might contribute to Parkinson's disease pathogenesis. However, even though these and other studies (Cuervo *et al.*, 2004; Martinez-Vicente *et al.*, 2008; Mak *et al.*, 2010) suggest that α-synuclein clearance and neurotoxic effects

Figure 5 Continued
(**B**) Representative nigral images of animals injected with GFP + Lamp2a (*top* row), GFP + α-synuclein (*middle* row), and α-synuclein + Lamp2a (*bottom* row) recombinant AAVs. The sections are counterstained with Nissl (blue) for anatomical reference. Scale bar = 200 μm. (**C**) Stereological quantification of numbers of TH+ neurons in the contralateral (C) and ipsilateral (I) side of the substantia nigra of all animals (*$P < 0.05$; **$P < 0.01$; $n = 12$/group). (**D**) Stereological quantification of the percentage loss of nigral TH+ neurons expressed as the mean % of the contralateral side in the GFP + Lamp2a-, GFP + α-synuclein-, and α-synuclein + Lamp2a-injected animals at 8 weeks post-injection (**$P < 0.01$; $n = 12$/group). (**E**) Representative striatal images of animals injected with GFP + Lamp2a (*top* row), GFP + α-synuclein (*middle* row), and α-synuclein + Lamp2a (*bottom* row) recombinant AAVs. Scale bar = 200 μm. (**F**) Densitometric analysis of dopaminergic fibre density in the striatum by TH immunoreactivity (**$P < 0.01$; ***$P < 0.001$; $n = 12$/group). (**G**) Quantification of striatal dopamine (DA) levels by high-performance liquid chromatography (*$P < 0.05$; $n = 12$/group). All data are expressed as the mean % of the contralateral side.

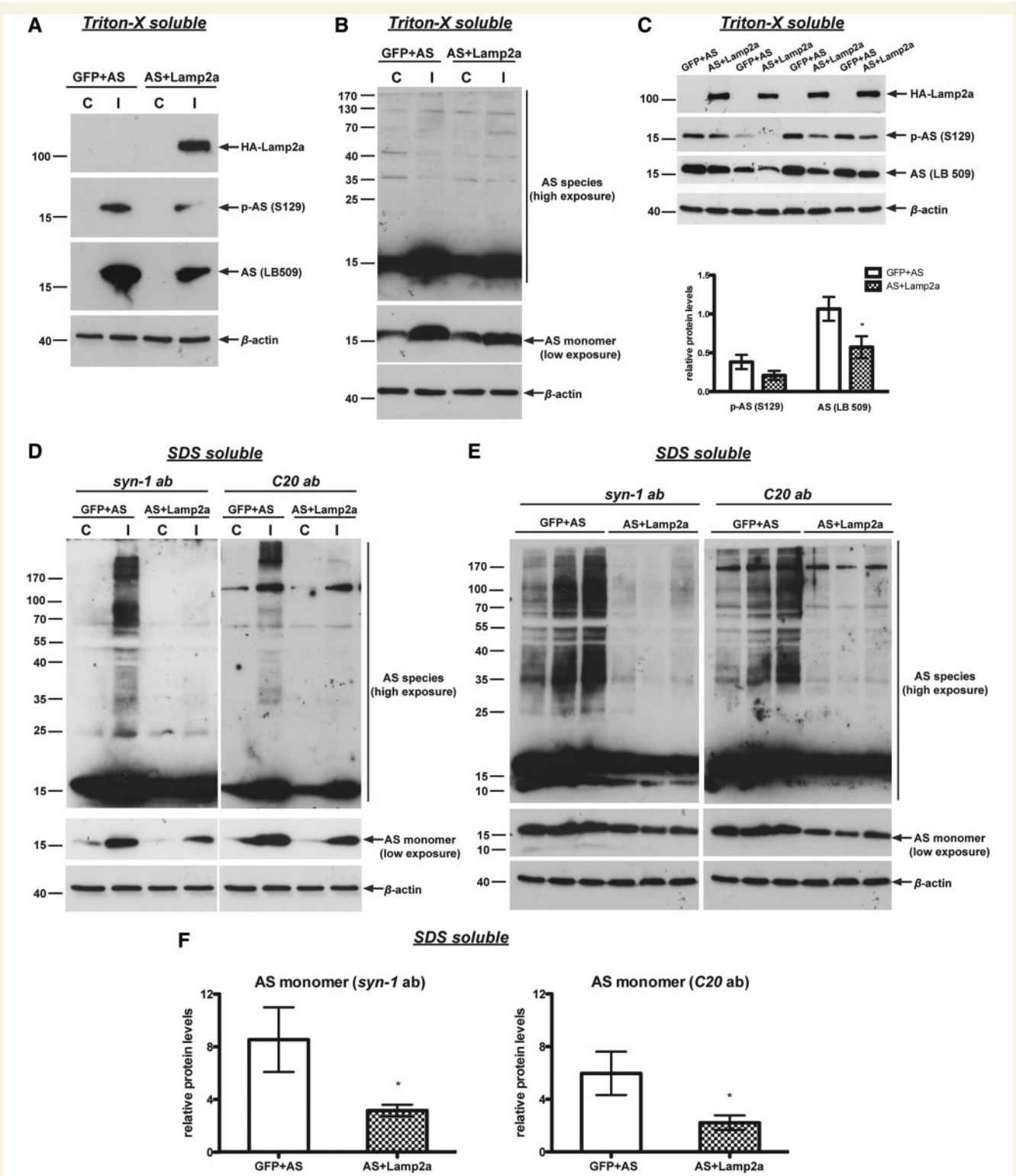

Figure 6 Augmentation of CMA-dependent proteolysis through Lamp2a upregulation decreases total human α-synuclein (AS) levels as well as the generation of Parkinson's disease-linked aberrant (high-molecular weight and phosphorylated) α-synuclein species. (**A**) Representative immunoblots for Lamp2a [haemagglutinin (HA) tagged], phosphorylated α-synuclein (p-AS), human α-synuclein (LB509 antibody), and β-actin (loading control) in the contralateral (C) and ipsilateral (I) side of GFP + α-synuclein and GFP + Lamp2a groups are shown. (**B**) Representative immunoblot for total (human and rat) α-synuclein protein levels probed with the C20 α-synuclein antibody in the Triton-X soluble midbrain fractions of GFP + α-synuclein-injected animals. The same immunoblot at high (total α-synuclein species) and low (α-synuclein monomer) exposure is shown. (**C**) Representative immunoblots for Lamp2a (HA tagged), p-α-synuclein, human α-synuclein (LB509 antibody), and β-actin (loading control) from four different animals/group are shown in the *upper* panel and

(continued)

are related to CMA, the *in vivo* function of CMA in Parkinson's disease pathogenesis remains elusive.

To explore further the role of CMA in α-synuclein pathobiology, we overexpressed Lamp2a together with wild-type α-synuclein in three different experimental models: human neuroblastoma cells, primary rat cortical neurons, and in the living brain. In human SH-SY5Y lines Lamp2a overexpression led to a similar increase of CMA-dependent proteolysis to that previously observed in cultured Chinese hamster ovary cells (Cuervo and Dice, 2000*a*). The Lamp2a-expressing lines displayed a higher turnover of α-synuclein, which was more evident in the high Lamp2a lines and under reduced serum conditions, indicative of increased α-synuclein degradation through induction of CMA in these lines. Importantly, the Lamp2a-expressing lines were specifically protected against adenoviral human α-synuclein-induced neurotoxicity in a Lamp2a-dose dependent-manner. The observed neuroprotection was likely mediated mainly through the upregulation of CMA-dependent proteolysis and subsequent accelerated clearance of CMA substrates other than α-synuclein, as total α-synuclein levels were not altered under these experimental conditions. One such CMA substrate could be myocyte enhancer factor 2D (MEF2D), as over-expression of both wild-type and A53T α-synuclein can inhibit MEF2D activity by interfering with its CMA-dependent degradation, leading to neuronal death, whereas mislocalized MEF2D was found elevated in A53T transgenic mice and in patients with Parkinson's disease (Yang *et al.*, 2009). The fact that the protection against α-synuclein-mediated neurotoxicity did not apply to other insults suggests that the induction of CMA does not activate a general survival pathway, but rather impacts specifically on α-synuclein-mediated pathogenic events.

To transfer these findings in a primary neuronal setting, we transduced rat cortical neurons with a high-titre adenovirus expressing Lamp2a, which increased CMA proteolysis by up to 3-fold. Co-transduction of a Lamp2a-expressing adenovirus together with a wild-type α-synuclein-expressing adenovirus resulted in the complete restoration of CMA activity and neuronal viability at early time points, in which the total steady-state levels of α-synuclein (endogenous and overexpressed) were unaltered. At later time points, Lamp2a and wild-type α-synuclein co-expression also reduced total α-synuclein burden, while continuing to provide protection against α-synuclein-mediated neurotoxicity. Thus, Lamp2a overexpression and the consequent induction of CMA in cortical neurons were sufficient to provide protection against α-synuclein-mediated neurotoxicity through two distinct mechanisms: restoration of lysosomal function and enhanced α-synuclein clearance.

To extend our findings *in vivo*, we utilized a well-established recombinant AAV-based model of Parkinson's disease-like neurodegeneration, in which human wild-type α-synuclein is overexpressed unilaterally in the rat substantia nigra. This model produces a gradual degeneration of dopaminergic neurons and their nigrostriatal projections, better resembling the human pathology than toxin-based models (Ulusoy *et al.*, 2010). Viral vector-mediated overexpression of α-synuclein also provides a model for cellular pathology, including the formation of α-synuclein-positive inclusions and α-synuclein dystrophic neurites in the nigrostriatal axis. Moreover, the unilateral expression of transgenes allows the uninjected contralateral side to act as an internal control for each animal. As therapeutic interventions with robust neuroprotection in toxin-based models of Parkinson's disease (Kirik *et al.*, 2001) have been far less effective in genetic α-synuclein models (Lo Bianco *et al.*, 2004; Decressac *et al.*, 2011) and clinical trials with patients with Parkinson's disease (Nutt *et al.*, 2003), we believe that the recombinant AAV-α-synuclein synucleinopathy model is better suited to yield clinically relevant results.

To establish this *in vivo* model, we first ensured that recombinant AAV-mediated Lamp2a nigral overexpression caused no detrimental effects to the nigrostriatal neurons. Even high titres of recombinant AAV-Lamp2a failed to induce neurodegeneration, suggesting that, at least for this time period, a high protein load of Lamp2a is not toxic. We then tested the effects of Lamp2a overexpression on recombinant AAV α-synuclein-mediated dopaminergic neurodegeneration. The observed α-synuclein-induced loss of TH+ nigral neurons and their striatal projections in the GFP+ α-synuclein-injected animals was within the expected range (Kirik *et al.*, 2002; Klein *et al.*, 2002; Yamada *et al.*, 2004). Strikingly, co-injection of α-synuclein + Lamp2a recombinant AAVs preserved completely nigral TH+ neurons, while at the same time restoring striatal dopamine levels and striatal TH+ fibre density to control levels. Assessment of a motor behaviour test revealed that GFP + α-synuclein-injected rats showed only a small, non-significant increase of amphetamine-induced rotations compared with GFP + Lamp2a controls. This was expected, as the recombinant AAV-α-synuclein model does not produce consistent behavioural deficits at the 8-week time point (Ulusoy *et al.*, 2010). Nonetheless, α-synuclein + Lamp2a-injected rats had a similar profile to controls, suggesting a reversal of this slight behavioural abnormality.

Biochemical analysis of the generated α-synuclein species revealed that co-injection of Lamp2a attenuated the accumulation of monomeric human-specific and phosphorylated (S129, p-α-synuclein) α-synuclein species in the Triton-X soluble ventral

Figure 6 Continued
quantification of p-α-synuclein and human-specific α-synuclein (LB 509) protein levels is shown in the *bottom* panel (*$P < 0.05$; $n = 12$/group). (**D**) Immunoblot depicting the accumulation of SDS soluble (Triton-X insoluble) high-molecular-weight α-synuclein species (stained both with monoclonal syn-1 and polyclonal C20 antibody) in the ipsilateral side (I) of GFP + α-synuclein- and α-synuclein + Lamp2a-injected animals. The same immunoblot at high (total α-synuclein species) and low (α-synuclein monomer) exposure is shown. (**E** and **F**) Representative immunoblots at high (total α-synuclein species) and low (α-synuclein monomer) exposure for syn-1 and C20 antibodies (**E**), as well as quantitative analysis (**F**) for monomeric α-synuclein (detected with syn-1 and C20 abs, low exposure) are shown (*$P < 0.05$; $n = 12$/group). Ab = antibody.

midbrain fractions, as well as the monomeric and high-molecular-weight α-synuclein species in the SDS-soluble fractions. It is important to note here that the effect on aberrant α-synuclein species achieved through CMA upregulation is commensurate to the general reduction of α-synuclein levels, and thus CMA induction appears to lead to a primary effect of increased clearance of monomeric, unmodified soluble α-synuclein, which secondarily leads to reduction of all other species. This would be consistent with the fact that the primary target for CMA appears to be the unmodified monomeric form of the protein (Martinez-Vicente *et al*., 2008). Overall, Lamp2a targeting facilitated the clearance of total and aberrant α-synuclein species in this *in vivo* recombinant AAV model of synucleinopathy. These findings are of particular importance since it is proposed that the tendency of α-synuclein to form oligomers and aggregates lies at the core of its pathological function.

Besides CMA, macroautophagy was also found to be responsible for the degradation of α-synuclein in various systems (Webb *et al.*, 2003; Lee *et al.*, 2004; Vogiatzi *et al.*, 2008), and there is extensive literature suggesting that the augmentation of macroautophagy may be beneficial in synucleinopathies and other neurodegenerative conditions (Sarkar *et al.*, 2009; Spencer *et al.*, 2009; Metcalf *et al.*, 2012). Activation of macroautophagy with rapamycin, which acts as an mTOR inhibitor, potentiated α-synuclein clearance and α-synuclein localization in acidic organelles in PC12 cells (Webb *et al.*, 2003). A protective role for the induction of macroautophagy against α-synuclein overexpression was described by Spencer *et al.* (2009) and Yu *et al.* (2009), whereas Cartier *et al*. (2012) showed that, in an α-synuclein transgenic mouse model, blocking UCHL1 function decreased α-synuclein levels, likely through the induction of macroautophagy. Moreover, rapamycin treatment in wild-type α-synuclein transgenic mice reduced α-synuclein inclusions through increased α-synuclein clearance; however, this was not accompanied by a conclusive protective effect (Klucken *et al.*, 2012). It is important to note that, unlike the experimental approach presented here, the induction of macroautophagy as a therapeutic strategy against synucleinopathies has not been tested in the nigrostriatal system, which is the primary site of neurodegeneration in Parkinson's disease.

Conversely, aberrant macroautophagy may have detrimental effects on cell survival. Overexpression of mutant A53T but not wild-type α-synuclein in PC12 cells led to a marked induction of macroautophagy, proteasomal and lysosomal dysfunction and autophagic cell death (Stefanis *et al.*, 2001). This upregulation of macroautophagy appears to be, at least in part, a secondary compensatory response following the primary defect at the level of CMA, whereas inhibition of such compensatory macroautophagy induction (pharmacologically or molecularly) promoted survival, suggesting that in this setting, the activation of macroautophagy was detrimental (Xilouri *et al.*, 2009). A recent report demonstrated that mutant α-synuclein exerted its toxicity in primary neurons through the induction of mitochondrial autophagy, providing a potential link between α-synuclein accumulation and mitochondrial dysfunction (Choubey *et al.*, 2011). These findings imply that the therapeutic targeting of macroautophagy for α-synuclein-related disorders should be applied with caution.

To our knowledge, this is the first study in which a potential therapeutic approach in this model of α-synuclein-mediated dopaminergic neurotoxicity conferred complete protection at the level of TH+ neurons and terminals, while achieving a simultaneous reduction of α-synuclein levels, especially of the aberrant high-molecular-weight and phosphorylated α-synuclein species. Thus, the results presented here represent the first successful attempt to reverse the toxicity associated with recombinant AAV-mediated nigral α-synuclein overexpression using a gene therapy approach. As such, the present study pinpoints, for the first time, an important *in vivo* role of CMA in wild-type α-synuclein pathobiology and provides further impetus for the idea of modulating the CMA pathway as a novel therapeutic approach in Parkinson's disease and related synucleinopathies.

Acknowledgements

The authors wish to acknowledge Björn Anzelius for the production of viral vectors, Anneli Josefsson, Ulla Samuelsson and Ulrika Sparrhult-Björk for technical support and Dr. Lee Clough for editing the manuscript for English style.

Funding

This work was supported by a Post-Doctoral Research Fellowship from the Parkinson Disease Foundation and a Target Validation grant from the Michael J. Fox Foundation to M.X. and L.S., by NEURASYNC: an Academic Industrial Initial Training Network (ITN) through the People program FP7 of the European Union and FCT (PTDC/SAU-NEU/105215/2008) grant to L.S. and D.K. and by FP7/REGPOT-2008-1 grant to L.S.. D.K. was supported by grants from the Swedish Research Council (2008-3092, 2009-2318) and the European Research Council (TreatPD 242932).

Supplementary material

Supplementary material is available at *Brain* online.

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