

TOM40 Mediates Mitochondrial Dysfunction Induced by α -Synuclein Accumulation in Parkinson's Disease

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Abstract

Alpha-synuclein (α -Syn) accumulation/aggregation and mitochondrial dysfunction play prominent roles in the pathology of Parkinson's disease. We have previously shown that postmortem human dopaminergic neurons from PD brains accumulate high levels of mitochondrial DNA (mtDNA) deletions. We now addressed the question, whether alterations in a component of the mitochondrial import machinery -TOM40- might contribute to the mitochondrial dysfunction and damage in PD. For this purpose, we studied levels of TOM40, mtDNA deletions, oxidative damage, energy production, and complexes of the respiratory chain in brain homogenates as well as in single neurons, using laser-capture-microdissection in transgenic mice overexpressing human wildtype α -Syn. Additionally, we used lentivirus-mediated stereotactic delivery of a component of this import machinery into mouse brain as a novel therapeutic strategy. We report here that TOM40 is significantly reduced in the brain of PD patients and in α -Syn transgenic mice. TOM40 deficits were associated with increased mtDNA deletions and oxidative DNA damage, and with decreased energy production and altered levels of complex I proteins in α -Syn transgenic mice. Lentivirus-mediated overexpression of Tom40 in α -Syn-transgenic mice brains ameliorated energy deficits as well as oxidative burden. Our results suggest that alterations in the mitochondrial protein transport machinery might contribute to mitochondrial impairment in α -Synucleinopathies.

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Introduction

Mitochondrial dysfunction is believed to play a pivotal role in the pathogenesis of Parkinson's disease (PD) [1,2]. Decreases in activity and protein concentration of electron transport chain (ETC) complex I are consistently found in brains and other tissues of PD patients [3,4,5]. Moreover, strong associations have been identified between genes causing familial PD syndromes and mitochondrial function [1]. Previous reports, including work from our group, showed significantly higher levels of somatic deletions in mitochondrial DNA (mtDNA) in individual post mortem dopaminergic neurons from PD and aged patients compared to non-PD or younger controls [6,7].

Alpha-synuclein (α -Syn) is an abundant presynaptic molecule [8] and a major component of Lewy bodies (LB) and Lewy neurites, which are neuropathological hallmarks of PD in the substantia nigra (SN) [9]. Its progressive intraneuronal aggregation has been proposed to play a central role in idiopathic PD and highlights its neurotoxic potential [10,11]. Mutations or multiplications in the gene encoding α -Syn cause autosomal dominant familial Parkinson syndromes (PARK1 and PARK4) [12,13]. Overexpression of human wt- α -Syn in transgenic (tg) mice leads to

motor deficits, dopaminergic loss, and formation of inclusion bodies, thereby mimicking certain aspects of a PD phenotype [14]. While it is yet not fully understood which are the mechanisms involved in α -Syn-mediated neuronal injury, an increasing body of evidence suggests that mitochondrial dysfunction plays an important role in this process [2,15,16]. Alpha-Syn was recently reported to localize to mitochondria [17,18,19]. Human α -Syn has a cryptic mitochondrial targeting sequence at its N-terminal domain that might facilitate its transport by the mitochondrial protein import machinery [20]. The close association between α -Syn and mitochondria is also reflected by the fact that α -Syn knockout mice are resistant to mitochondrial damage caused by complex I inhibitors, which usually lead to a PD-like phenotype in mammals and rodents [21,22].

In the present study we investigated α -Syn-induced mitochondrial toxicity. We show that α -Syn causes mitochondrial dysfunction and DNA damage *in vitro* as well as *in vivo*. We present evidence showing that oxidative stress and impaired mitochondrial function are mechanisms contributing to this damage, mediated mainly by a decrease in the mitochondrial outer membrane transport protein, TOM40 (translocase of the outer mitochondrial membrane). Overexpression of TOM40 in α -Syn transgenic mice

successfully ameliorated the mitochondrial deficits, suggesting it might represent a novel target for PD therapies.

Materials and Methods

Ethics Statement

Experiments involving human samples were approved by the Institutional Review Boards of the University of Munich as well as the German Brain Bank, BrainNet (Permit Number: GA44), in Munich. All investigations involving human samples have been conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all participating subjects, where appropriate.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of California San Diego (UCSD, Permit Number: S02221). All surgery was performed under anesthesia, and all efforts were made to minimize suffering.

Animals

For this study we used heterozygous transgenic mice (Line D) expressing wild type human α -Synuclein under the regulatory control of the platelet-derived growth factor- β (PDGF β) promoter [14] ($n = 15/\text{group}$, 5 months-old; 8 females, 7 males). These mice develop intra-neuronal accumulation of α -Syn aggregates throughout the hippocampus and neocortex, similar to what is observed in patients with PD and Lewy-Body disease [14].

Tissue Processing and Immunohistochemistry

Following NIH guidelines for the humane treatment of animals, mice were sacrificed under anesthesia and brains were removed. The right hemisphere was immersion-fixed in 4% paraformaldehyde in pH 7.4 phosphate-buffered saline and serially sectioned at 40 μm with a vibratome (Leica, Deerfield, Illinois, USA). The left hemisphere was immediately snap frozen in liquid nitrogen and kept at -80°C until further processing.

For laser capture-microdissection (LCM) of α -Syn-positive or α -Syn-negative neurons, 10 μm thick cryosections including the hippocampus and posterior cortex were cut on special membrane slides (Leica Microsystems, Germany). Sections were briefly fixed in ice-cold acetone and double-labeled with fluorescently-conjugated primary antibodies for anti-NeuN (1:200, Chemicon, Temecula, CA, USA) and anti- α -Syn (1:100, Chemicon, Temecula, California, USA). Fluorescent labeling of the anti- α -Syn-antibody was done with the Alexa Fluor[®] 488 (green) Monoclonal Antibody Labeling Kit while the anti-NeuN-antibody was labeled with Alexa Fluor[®] 594 (red; both Molecular Probes, Eugene, OR, USA). Primary antibodies were incubated for 1 hour at room temperature followed by dehydration in alcohol and air drying for 15 minutes. LCM of neurons with and without intensive α -Syn-immunostaining was performed on a Leica LMD 6000 LCM (Leica, Wetzlar, Germany). About 20 neurons per animal were picked from posterior cortex adjacent to the hippocampus and collected in one PCR tube. Samples were collected in triplicate.

Fixed brain sections were used for the immunostaining of markers of oxidative DNA damage (8-Hydroxy-2'-deoxyguanosine, 8-OHdG, 1:200), TOM40 and TOM20 (both from Santa Cruz Biotechnology, Santa Cruz, CA, at 1:250) and α -Syn-accumulation (1:1,000, both Chemicon, Temecula, California, USA). Primary antibodies were incubated overnight, followed by detection with species-appropriate secondary antibodies (1:2000,

Vector Laboratories, Burlingame, Ca, USA) and developed with 3,3'-Diaminobenzidine (DAB). Sections were transferred to SuperFrost slides (Fisher Scientific, California, USA) and mounted with anti-fading media (Vector Laboratories).

Detection and Quantification of mtDNA Deletions and mtDNA Copy Number

For mtDNA analysis of brain homogenates, the posterior half of frozen hemibrains was homogenized with 20 strokes of a Dounce homogenizer in 200 μl of cold PBS. Total DNA was extracted from half of this homogenate (DNeasy Blood & Tissue Kit, Qiagen, Hilden, Germany), the other half was used for RNA extraction. DNA concentration and purity was determined by UV-spectrophotometry at 260/280 nm. For the analysis of single cells after LCM, mtDNA was extracted with QIAmp DNA Micro Kit (Qiagen) according to the manufacturer's protocol and eluted in 20 μl of ddH₂O.

Low-abundance mtDNA deletions were detected by a two-step nested long-range PCR protocol (Expand Long Range dNTPack[®], Roche, Mannheim, Germany) to amplify a final ~ 10 kb fragment of the deletion-prone "major arc" of the mitochondrial genome as we previously reported [6,23]. In brief, first round PCR reactions were done on 10 ng of template DNA under standard conditions and using 30 pmol each of forward (mtDNA nt 4796-4817) and reverse (mtDNA nt 16020-16001) primers. Amplifications were carried out using a GeneAmp[®] PCR System 9600 (Applied Biosystems, Carlsbad, CA, USA) with the following cycling conditions: 3 minutes at 93°C ; 10 cycles of 93°C for 30 sec, 58°C for 30 sec, 68°C for 12 min; 20 cycles of 93°C for 30 sec, 58°C for 30 sec, 68°C for 12 min +5 sec per additional cycle; final extension of 11 min at 68°C . This reaction product was then diluted 1:50 and 1 μl was used as template in a second round of PCR amplification under similar conditions but with primers mtDNA nt 5591–5610 and mtDNA nt 15221–15000. Amplified products were separated through 0.8% agarose gels containing ethidium bromide and visualized over UV light.

The levels of mtDNA deletion (percentage of total mtDNA affected by deletions) were quantified by a real-time PCR assay, which we previously established for human mtDNA [24,25], based on the differential detection of DNA regions comprised in the deletions and that serves as target sequences for amplification. We designed three sets of primers and sequence specific probes (FAM/MGB-labeled TaqMan[®] probes, Applied Biosystems), which span the mtDNA molecule with roughly the same distance between them. Primers covered the ND1-gene nt3112-3136 (F) and nt3210-3176 (R), and probes were nt3154-3169; COX3 gene, nt8921-8945 (F) and nt8998-8974 (R) and cytochrome b (Cytb) gene, nt14614-14639 (F) and nt14718-14694 (R). Real-time PCR reactions were performed with TaqMan[®] Gene Expression Master Mix (Applied Biosystems) and 50ng of template DNA extracted from brain homogenates, or 2 μl of total DNA in the case of single cell experiments. Amplification conditions were 2 min at 50°C , 10 min at 95°C then 40 cycles of 15 s at 95°C and 1 min at 60°C . PCR reactions were performed on a StepOne Plus 96-well format system (Applied Biosystems). Deletion levels (in % of total mtDNA) were calculated using relative quantification according to the formula $(1 - 2^{-\Delta\Delta C_t}) * 100$ as we previously reported in more detail [24]. Relative mtDNA copy number was determined by comparing the highest quantity of the three mtDNA genes to a nuclear endogenous control gene (β -Actin, ACTB TaqMan[®] assay, Applied Biosystems).

Tissue Extraction, Isolation of Mitochondria and Quantification of ATP

Frozen anterior hemibrains were homogenized by sonication in 200 μ l ice cold PBS and immediately snap frozen in liquid nitrogen and stored at -80°C . 5 μ l of this homogenate was used for quantification of protein concentration by BCA protein assay (Pierce, Rockford, IL, USA). Quantification of ATP levels was done with a luciferase assay (CellTiter-Glo[®], Promega, Madison, WI, USA) and normalized by protein. All experiments were performed in triplicate.

Intact mitochondria were isolated from anterior hemibrains by differential centrifugation (Mitosciences, Eugene, OR, USA). As recommended, isolations were performed on ice and buffers were supplemented with phosphatase and protease inhibitors (Roche). Proteins were extracted from isolated mitochondria with RIPA lysis buffer and centrifugation for 10 minutes at 10,000 RPM.

Western Blot Analysis

TOM40, TOM20 and respiratory chain subunit protein levels were determined by immunoblot analysis from isolated mitochondria. Briefly, 20 μ g of total extracted mitochondrial protein were resolved by electrophoresis in 12% Bis-Tris SDS-PAGE gels (Invitrogen, Carlsbad, CA, USA) and transferred onto Immobilon membranes. Primary antibodies against subunits of all five respiratory chain complexes (MitoProfile[®] Total OXPHOS rodent Antibody Cocktail, Mitosciences, Eugene, OR, USA) or against TOM40 or TOM20 (both Santa Cruz Biotechnology) were all incubated in a 1:1,000 dilution overnight at 4°C and developed on a VersaDoc gel-imaging system (Bio-Rad, Hercules, CA). Beta-actin levels, detected by anti-ACTB antibody (1:1,000, Sigma), were used to normalize loading. The Quantity One software package (Bio-Rad, Hercules, CA) was used for densitometry analysis.

In vitro Analysis of TOM40 Levels and Oxidative Stress Markers on α -Syn-Overexpression B103 System

B103 rat neuroblastoma cells were grown under standard conditions in DMEM supplemented with 10% FBS and 1x PenStrep (Life Technologies) and incubated at 37°C with 5% CO_2 -supplemented air. Cells were infected with lentiviral vectors encoding human wild-type α -Syn or A53T or A30P mutated isoforms; TOM40 or empty backbone as control. Cells grown on coverslips were fixed 48 h after infection with 4% paraformaldehyde for immunohistochemical analysis.

Mitochondria were labeled in vivo by incubation with 1 mM MitoTracker (Life Technologies) in serum-free DMEM for 45 min. Detection of ROS production was performed in vivo with CellROX Deep Red Reagent 5 μM (Life Technologies) for 45 minutes (co-incubation with MitoTracker). This reagent yields a red cytoplasmic signal upon oxidation.

Quantification of Oxidative Damage to DNA by ELISA

DNA oxidative lesions were quantified by ELISA. In brief, DNA was dissociated into single-strands by incubation at 95°C for 5 minutes and rapidly chilling on ice. DNA was digested to nucleosides with nuclease P1 (15 U, Sigma) followed by treatment with 8 units of alkaline phosphatase (Sigma) for 1 hr at 37°C . 8-OHdG quantification was then performed with OxiSelect[®] Oxidative DNA Damage ELISA Kit (Cell Biolabs, San Diego, CA, USA) as per manufacturer's instructions.

Lentivirus Construction for Delivery and Overexpression of TOM40

Murine TOM4 cDNA (clone #3326417, OpenBiosystems) was PCR amplified to include BamHI and ApaI restriction sites. This was then cloned into the 3rd generation lentivirus vector to generate the LV-TOM40 virus. The LV-control virus consists of the same backbone containing the CMV promoter without a transgene cloned downstream of promoter. Lentiviruses were prepared as previously described by transient transfection in 293T cells [26].

Stereotaxic Delivery of Lentiviruses to α -Syn Tg Mice

α -Syn tg mice and their non-transgenic litter mates ($n = 8$ /group, 10 months old) received stereotaxic injections of 3 μ l of the lentiviral preparations (2.5×10^7 TDU) into the hippocampus and cortex as previously described [27]. Mice were anesthetized and received quadrilateral injections of either LV-TOM40 ($n = 8$, 4 α -Syn tg, 4 non-tg), or LV-control ($n = 8$, 4 α -Syn tg, 4 non-tg) on a Kopf stereotaxic apparatus. Coordinates for the hippocampus: AP -2.0 mm, lateral 1.5 mm, depth 1.3 mm; and for the cortex: AP -0.5 mm, lateral 1.5 mm and depth 1.0 mm. Four weeks after the lentiviral injection, mice were anesthetized with chloral hydrate and flush-perfused transcardially with 0.9% saline. Brains were removed and the right hemibrain was post-fixed in phosphate-buffered 4% PFA (pH 7.4) at 4°C for 48 hours for neuropathological analysis, while the left hemibrain was snap-frozen and stored at -80°C for subsequent ATP and protein analysis.

Stereological Analysis of TOM40, α -Syn, and 8-OHdG on Brain Sections

Analysis of α -Syn accumulation was performed in serially-sectioned, free-floating, blind-coded vibratome sections from tg and non-tg mice treated with LV-TOM40 and control vectors. Sections immunolabeled with antibodies against α -Syn and NeuN (three from each mouse at 100 μm intervals) were analyzed by the disector method using the Stereo-Investigator System (MBF Bioscience, Williston, VT) and the results were averaged and expressed as numbers per mm^3 . Sections immunolabeled with antibodies against TOM40 and GFAP were imaged with an Olympus X digital microscope (BX51) and analyzed with the ImageQuant program. Results are expressed as corrected optical density. Sections immunolabeled with antibodies against 8-OHdG were serially imaged with the laser scanning confocal microscope (LSCM, MRC1024, BioRad) and analyzed with the Image 1.43 program (NIH), as previously described [28,29]. For each mouse, a total of three sections were analyzed and results expressed as pixel intensity. All sections were processed simultaneously, under the same conditions.

All experiments were done blind coded and in triplicate. Values in the figures are expressed as means \pm SEM.

Human Brain Samples

Human midbrain samples from five PD cases (75.3 ± 12.8 years) and five age-matched non-neurodegenerative controls (69.1 ± 15.4 years) were provided by the German brain bank BrainNet[®] following ethical approval by the Institutional Review Board (ethics committee) of the University of Munich. Frozen brain sections were cut with a cryostat and two 25 μm thick sections were collected per brain. Protein extraction followed the same protocol as described above for mouse brain sections.

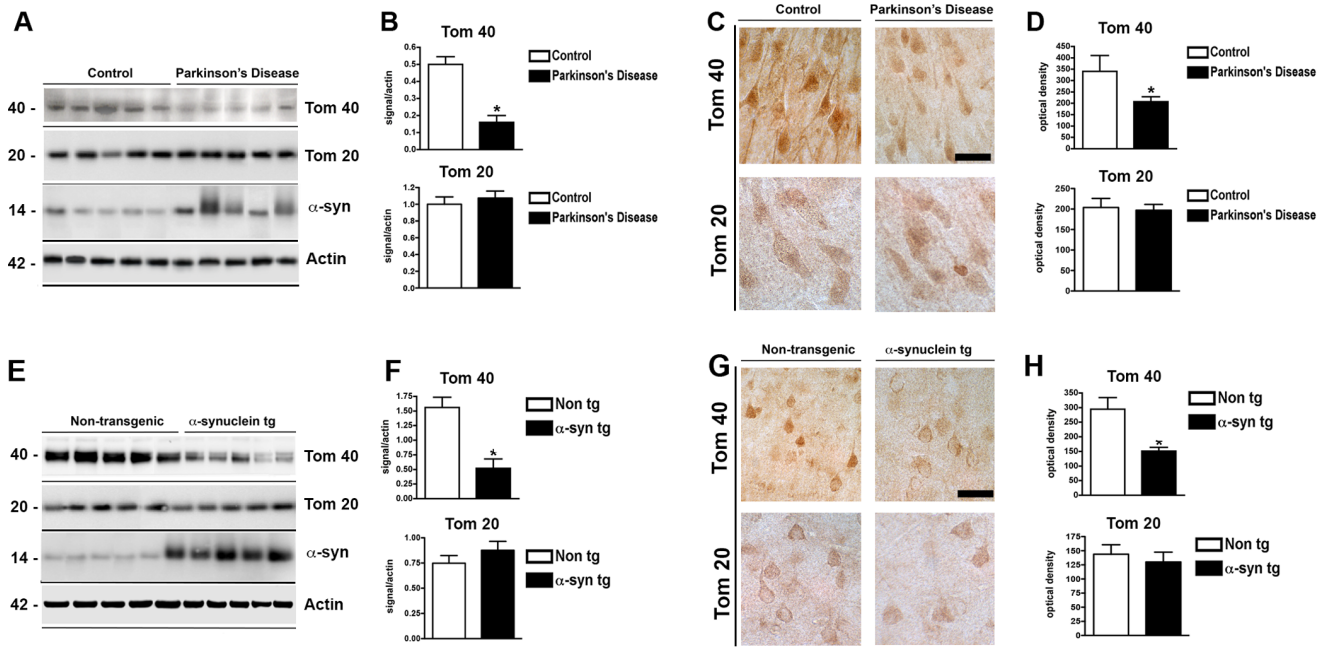


Figure 1. TOM40 protein levels are decreased in PD brains and in α -Syn transgenic mice. (A) Western blot analysis showing levels of mitochondrial proteins TOM40 and TOM20 as well as α -Syn on postmortem human brain samples from control subjects and PD patients (β -Actin signal is shown as normalization control). (B) Densitometric analysis of TOM40 and TOM20 immunoreactivity. (C) Immunohistochemical detection of TOM40 and TOM20 in control and PD brains. (D) Optical density analysis of TOM40 and TOM20 immunoreactivity. (E) Western blot analysis of Tom40, Tom20 and human- α -Syn levels on whole brain homogenates from α -Syn-tg mice and non-transgenic littermate controls. (F) Densitometric analysis of Tom40 and Tom20 on mice brains. (G) Immunohistochemical detection of Tom40 and Tom20 in α -Syn-tg and non-transgenic mice. (H) Optical density analysis of Tom40 and Tom20 on mice brains. * $p < 0.05$ per Student's *t* test. Scale bar represents 25 μ m. doi:10.1371/journal.pone.0062277.g001

Statistical Analysis

Statistical analysis was performed with the SPSS 12.0 software package (SPSS Inc., Chicago, IL, USA). The Kolmogorov-Smirnov Test was used to check for normal distribution of the data, in which case *t*-tests were applied to identify significant ($p < 0.05$) group differences. For multiple comparisons, the one-way ANOVA with pos-hoc Fisher tests was used. All data proved to be normally distributed.

Results

TOM40 Protein Levels are Altered in PD and α -Syn-transgenic Mouse Brains

α -Synuclein is present at synaptic terminals and can be identified at both the cytoplasmic and nuclear compartments. In addition, α -Syn has been reported to localize to mitochondria, and its translocation is presumably mediated by the mitochondrial import machinery, after recognition of a mitochondrial targeting sequence present in α -Syn [20]. The translocase of the outer mitochondrial membrane (TOM) is a component of the import machinery, and TOM40- the transmembranous domain of the TOM complex- was reported to directly interact with α -Syn and to be involved in its import into mitochondria [20,30]. We hypothesized that α -Syn accumulation might induce alterations in TOM40 that could impair mitochondrial function. To address this question we first investigated TOM40 protein levels in postmortem midbrain samples from PD patients. Compared to age-matched control subjects, PD brains showed a significant decay in TOM40 levels (Figure 1A–D). In addition we evaluated the levels of the mitochondrial import receptor TOM20, another component of the TOM40 complex that participates in recogni-

tion of targeting sequences tethering the pre-sequence to the complex and increasing import efficiency [31]. No alterations were observed in TOM20 levels in PD brains, suggesting that the observed alterations on TOM40 are specific rather than a consequence of general decay of mitochondrial proteins (Figure 1A–D). PD cases included in the present study were carefully selected based on neuropathological examination postmortem, and we further characterize them here by quantification of α -Syn levels. All cases examined showed significant accumulation of α -Syn, with 3 (out of 5) showing increased aggregation of α -Syn (Figure 1 A). Decay of TOM40 levels corresponded to α -Syn accumulation, suggesting a functional link among these events. Moreover, decreased levels of TOM40 protein were also observed in the brains of the α -Syn transgenic mouse model of PD, when compared to non-transgenic littermates, with no overt changes on TOM20 levels (Figure 1 E–H), decays that also corresponded with α -Syn-overexpression.

TOM40 is Altered in Wild-type and A53T- α -Syn Accumulating Cells

The pathological hallmark of PD is the accumulation of α -Syn in Lewy Body structures [9]. Two dominant mutations in α -Syn, A53T and A30P cause familial early onset PD [13,32]. We therefore investigated if the accumulation of wild type α -syn and its most common mutated forms results in Tom40 decrease thus impairing mitochondrial function. We generated an *in vitro* system by infection of B103 rat neuroblastoma cells with lentiviral constructs harboring human wild type α -Syn (LV- α -syn), mutations A53T (LV- α -syn-A53T) or A30P (LV- α -syn-A30P), Tom40 (LV-Tom40) or a combination of these constructs. Overexpression of wild-type α -syn and A53T mutant resulted in specific decay of

Tom40, while Tom20 remained unaltered (Figure 2 A–B). Interestingly, A30P mutation did not alter Tom40 levels. Both mutations are located at the N-terminal fragment of α -Syn, in the proximity of the encrypted mitochondrial signal. We speculate that A30P mutation might alter the affinity of α -Syn for the mitochondrial membranes, and therefore might not interact with Tom40. We next overexpressed Tom40 on α -Syn-accumulating cells by co-infection of B103 cells with LV-Tom40 (Figure 2 C–E). Immunohistochemical detection of α -Syn on double-infected cells showed increased Tom40 was able to reduce the accumulation of α -Syn, both wild-type and A53T isoforms, while no effects were observed on A30P (Figure 2 D–E). These results suggest a complex regulatory feedback mechanism by which Tom40 and α -Syn regulates the levels of each other. We hypothesize that, in the case of Tom40 overexpression, restored mitochondrial function will decrease oxidative stress preventing further misfolding of α -Syn and might also contribute to redistribution and/or degradation of protein aggregates.

Tom40 Alterations Induced by α -Syn Accumulation Results in Increased Oxidative Damage and DNA Deletions on the Mitochondrial Genome

We further evaluated if the observed reductions on Tom40 associated with accumulation of wild-type α -Syn and A53T might increase oxidative stress. Overexpression of wild-type and A53T- α -Syn, and to a lesser extent of A30P α -Syn, resulted in reduced mitochondrial content as evidenced by Mitotracker labeling (Figure 3 A–B), probably due to increased mitochondrial autophagy in response to α -Syn accumulation as reported earlier [33]. In addition, α -Syn overexpression increased ROS production in the cytoplasm (Figure 3 C–D) and higher levels of

oxidative DNA lesions, evidenced by higher 8-OHdG (8-hydroxy-2'-deoxyguanosine) levels (Figure 3 E–F). Importantly, overexpression of Tom40 on α -Syn-accumulating cells helped preserve mitochondrial integrity, with concomitant reductions on ROS production and oxidative DNA damage, in a support of a functional relation between α -Syn, Tom40 and mitochondria (Figure 3 A–F).

An important body of evidence from genetic analysis, post-mortem human brain tissue examination and studies on animal models strongly suggest that mitochondrial dysfunction is a key pathological mechanism in PD, mainly leading to increased oxidative stress [34]. After our observations showing increased oxidative DNA lesions in α -Syn-accumulating cells, we analyzed 8-OHdG levels in α -Syn transgenic mice brains. Immunohistochemical analysis showed increased intensity of 8-OHdG staining in cortical areas of α -Syn transgenic mice, when compared to non-transgenic littermates. These results were supported by ELISA-based quantification of 8-OHdG on brain homogenates (Figure 4 A and B).

In addition to generation of reactive oxygen species (ROS) and oxidative stress, mitochondrial dysfunction results in damage and deletions to mitochondrial DNA (mtDNA), which is particularly susceptible to ROS generated by the respiratory chain due to its physical proximity. We have previously shown large-scale mtDNA deletions in postmortem human brain samples from patients diagnosed with PD [6]. Therefore, we measured the levels of mtDNA deletions in α -Syn transgenic mice. We detected large-scale mtDNA deletions by long-extension PCR in brain homogenates from both transgenic animals and non-transgenic littermates (Figure 4 C). Analysis of the resulting amplicons showed a higher number of partial deletions in mtDNA on

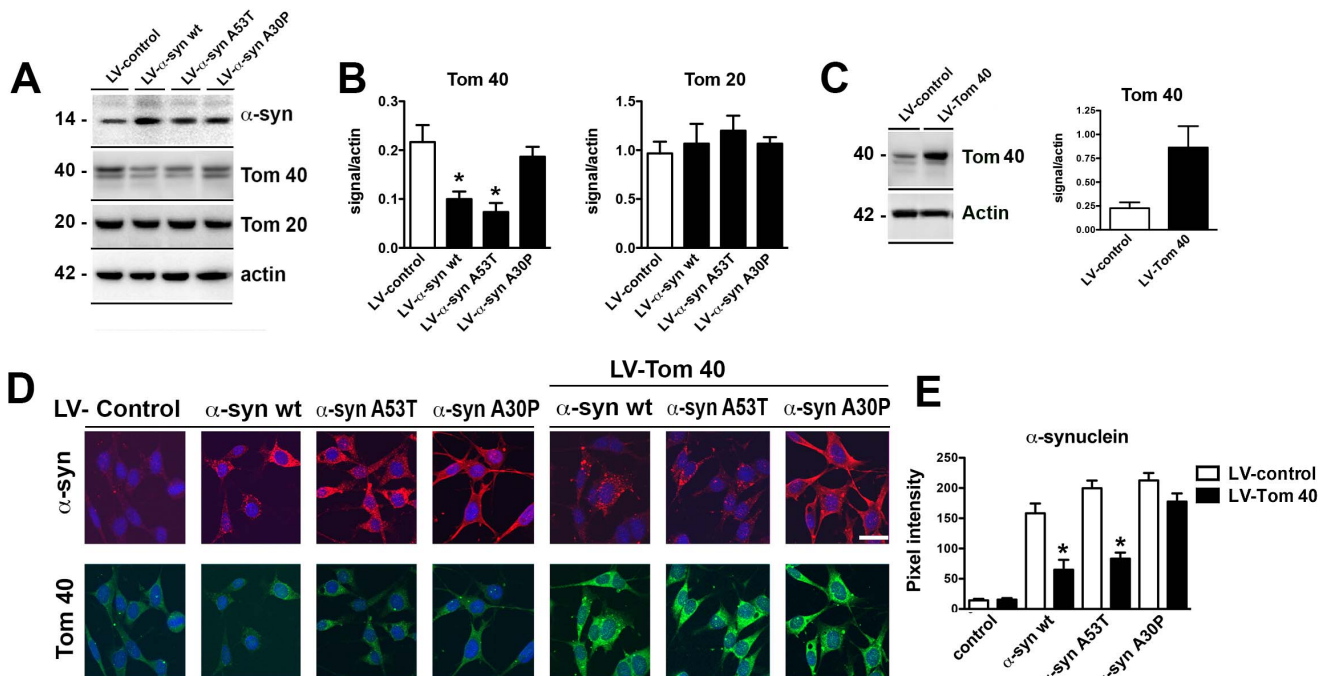


Figure 2. Tom40 is reduced in B103 cells overexpressing wild type and A53T- α -Syn. B103 neuroblastoma rat cells were infected with lentivirus encoding human α -Syn wild type (LV- α -Syn) or its mutated isoforms A53T (LV- α -Syn-A53T) and A30P (LV- α -Syn-A30P); Tom40 (LV-Tom40) or a combination of these vectors. (A) Western blot detection of α -Syn, Tom40 and Tom20 on whole cell lysates from infected B103 cells. (B) Densitometric analysis of Tom40 and Tom20 levels. (C) Efficient overexpression of Tom40 is achieved in B103 cells by infection with LV-Tom40. (D) Immunohistochemical detection of α -Syn and Tom40 in B103 cells infected with the corresponding lentiviral constructs. (E) Pixel intensity analysis of α -Syn levels, showing reduction of α -Syn in Tom40-overexpressing cells. * $p < 0.05$ per Student's t test. Scale bar represents 10 μ m. doi:10.1371/journal.pone.0062277.g002

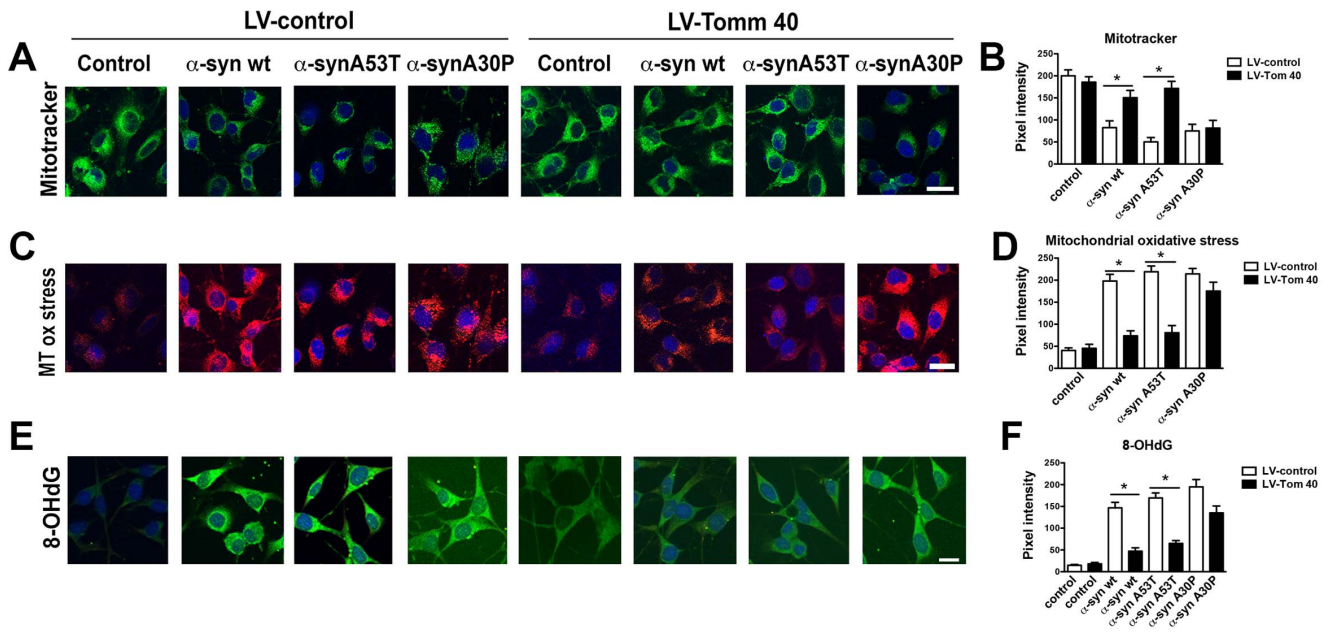


Figure 3. Mitochondrial impairment and oxidative stress induced by α -Syn-accumulation is reduced by overexpression of Tom40. B103 neuroblastoma rat cells were infected with lentivirus encoding human α -Syn wild type (LV- α -Syn) or its mutated isoforms A53T (LV- α -Syn-A53T) and A30P (LV- α -Syn-A30P); Tom40 (LV-Tom40) or a combination of these vectors. (A) Immunohistochemical detection of mitochondria by in vivo labeling with MitoTracker. (C) In vivo detection of mitochondria-derived reactive oxygen species (ROS) in cytoplasm by CellROX fluorogenic probes. (E) Fluorescent immunolabeling of oxidative DNA lesions (anti-8-OHdG). (B, D and F) Pixel intensity analysis of the corresponding immunosignals. Scale bar represents 10 μ m. * p <0.05 per Student's *t* test. doi:10.1371/journal.pone.0062277.g003

transgenic mice, i.e. higher number of shorter bands than intact mitochondrial genome (approx. 10 Kb), which was better conserved in non-transgenic littermates. In order to determine the actual deletion load within the whole brain and within specific neuronal populations, we applied real-time PCR analysis (qPCR). Increased percentage of mtDNA deletions was confirmed on brain homogenates from α -Syn transgenic mice in comparison to control animals (Figure 4 D). We next analyzed if α -Syn accumulation correlates with mtDNA damage at the single neuron level. Laser-capture microdissection (LCM) of cortical neurons showing either intense α -Syn staining (Figure 4 E, green labeling) or those testing negative for α -Syn-immunoreactivity was followed by qPCR determination of mtDNA deletions. Neurons accumulating α -Syn contained a higher deletion load than neighboring neurons of the same brain region without intense α -Syn immunostaining (Figure 4 F). As a reference, the ratio between mitochondrial and nuclear DNA copy number remained unchanged in both neuronal populations sampled. These results strongly link α -Syn accumulation to the induction of important deletions in mtDNA.

The human mitochondrial genome encodes a total of 37 genes, including subunits of the complexes I, III, IV and V [35]. We investigated the effects of α -Syn accumulation on the expression of mitochondrial proteins. At the protein level, α -Syn transgenic mice showed a significant decrease of the complex I subunit NDUFB8 (Figure 4 G–H), while subunits of the other 4 oxidative phosphorylation (OXPHOS) complexes appeared unaltered. These alterations in protein expression are expected to have a profound impact on mitochondrial function.

Lentiviral-mediated Delivery of Tom40 Reverses α -Synuclein-induced Mitochondrial Toxicity

Taken together, the above results suggest that impairment of mitochondrial protein import is an early event that greatly

contributes to α -Syn toxicity. To test this hypothesis, we delivered a lentiviral vector expressing Tom40 to the hippocampus of α -Syn transgenic and control animals. Intracranial lentivirus injection resulted in increased levels of Tom40 throughout the hippocampus and dentate gyrus (Figure 5 A and B). Overexpression of Tom40 resulted in protection against oxidative DNA lesions induced by α -Syn accumulation, as evidenced by reduced 8-OHdG levels in α -Syn tg mice that approached those observed in non-transgenic control animals (Figure 5 C and D). Four weeks after lentiviral delivery of Tom40, ATP levels were increased in both, α -Syn tg and control animals, suggesting a significant improvement of mitochondrial function (Figure 5 E).

Overexpression of Tom40 Results in Redistribution of α -Synuclein in Transgenic Mice Brains

Tom40 overexpression resulted in an increased of NeuN positive cells in transgenic animals, which reached similar levels to those observed in non-transgenic control mice (Figure 6 A and C). In addition, α -Syn tg mice that received Tom40 lentiviral injections showed reduced glial cell counts than the animals injected with control virus (Figure 6 B and D). These results suggest that Tom40 gene therapy was able to diminish neurodegenerative and inflammatory processes in brains of α -Syn tg mice. As we previously observed in our *in vitro* cellular model, overexpression of Tom40 appears to decrease α -Syn accumulation, which now redistributes partly from cell bodies into the axons, mirroring the physiological location of α -Syn into synaptic terminals [36] (Figure 6 E and F), an effect that might be associated to restored mitochondrial function.

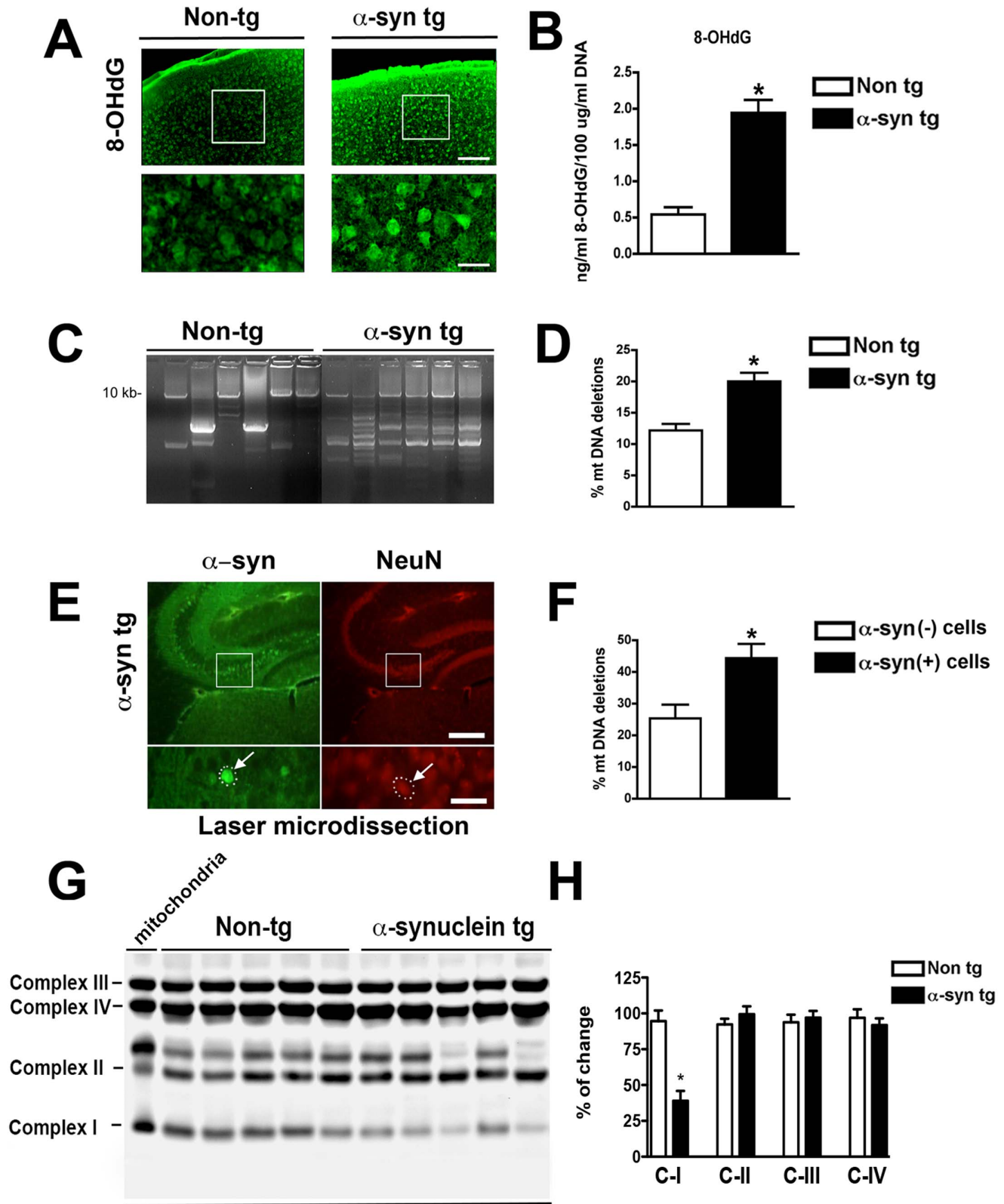


Figure 4. Alpha-synuclein overexpression results in oxidative DNA damage, alterations in respiratory chain complex I and mitochondrial DNA deletions in transgenic mice brains. (A) Immuno-histochemical detection of 8-OHdG suggesting increased oxidative DNA damage in α -Syn tg mice brains. (B) 8-OHdG levels were quantified on whole brain in α -Syn tg and non-transgenic control mice by ELISA. Scale bar represents 25 μ m. (C) Long extension PCR showing large-scale mtDNA deletions in α -Syn tg mice. (D) Quantitative determination of mtDNA deletions by real-time PCR showing increased mtDNA deletion levels in the brain of α -Syn tg mice compared to control animals. (E) Immunostaining for α -Syn (green signal) and NeuN (red signal; arrow marks the identical neuron in double labeling) used for Laser Capture Microdissection (LCM). Scale bar

represents 250 μ m in the upper panel and 25 μ m in the close-up lower panel. (F) Quantification of mtDNA deletion levels by real-time PCR on individual neurons with either intense or negative α -Syn immunoreactivity after LCM. (G) Western blot analysis of mitochondrial OXPHOS in brain homogenates from α -Syn tg and wt mice. Lane 1 shows mitochondrial proteins standard marker. (H) Densitometric analysis of the levels of Complexes I, II, III and IV. * p <0.05 per Student's t test. doi:10.1371/journal.pone.0062277.g004

Discussion

Understanding the mechanisms of α -Syn-mediated neurotoxicity and neurodegeneration is crucial for the development of specific therapies for highly prevalent neurological diseases, such as PD. Here we provide new evidence for a possibly under-recognized pathomechanism by which extra-mitochondrial α -Syn could interfere with mitochondrial function. We report here a specific decrease in TOM40 protein levels both in mouse brain homogenates as well as in human midbrain samples, inversely associated with α -Syn accumulation. We show that, in addition to wild type α -Syn, A53T- α -Syn also alters Tom40 levels, while accumulation of A30P mutant has little effect on this system. Reduction on Tom40 results in higher ROS production; increased oxidative damage to the DNA accompanied by higher level of mtDNA deletions; altered mitochondrial integrity and reduced ATP production. Overexpression of Tom40 on α -Syn-accumulating neuronal cells partially restores mitochondrial function, reducing the oxidative burden and also impacting α -Syn aggregation/accumulation. Although it is unclear how α -Syn could promote the reduction in TOM40 levels, we found a highly significant negative correlation between α -Syn and TOM40 by performing data mining on expression levels derived from 381 human blood samples (data not shown) suggesting that α -Syn is a negative regulator of TOM40 either directly or by indirect mechanisms.

It is an open question by which mechanisms α -Syn promotes its neurotoxicity and especially how it impacts mitochondria. Direct interaction of α -Syn with intra-mitochondrial proteins, including the OXPHOS complexes have been proposed [37]. It has been recently shown that α -Syn is able to enter mitochondria via the import channels by means of a mitochondrial targeting sequence [20,37]. Additional work confirmed the intra-mitochondrial localization of α -Syn, which, interestingly, was more abundant in SN than in other brain regions in rat models [38]. Similar findings were also reported in post mortem brain samples from PD patients [20]. α -Syn interacts with TOM40, which is the channel-forming β -barrel subcomplex and an essential part of the mitochondrial import machinery [30,39]. Interestingly, mitochondrial localization of α -Syn is inhibited by antibodies directed against TOM40 [20]. Inside mitochondria, α -Syn was shown to interact with and to inhibit complex I, resulting in increased oxidative stress [17,19,20,40], as we observed in the present study.

Mutations on α -Syn, in particular dominant mutations A53T and A30P, have been associated with familial PD manifestations [13,32]. These mutated isoforms of α -Syn are able to form aggregates and to trigger neurotoxicity [19], and seem to increase the shuttling of α -Syn between the nucleus and the cytoplasm [41]. We investigated the effects of wild type and mutated α -Syn on Tom40 and present evidence here showing that wild type and A53T mutations both specifically reduce Tom40 levels and alter mitochondrial integrity and functioning, as evidenced by reduced

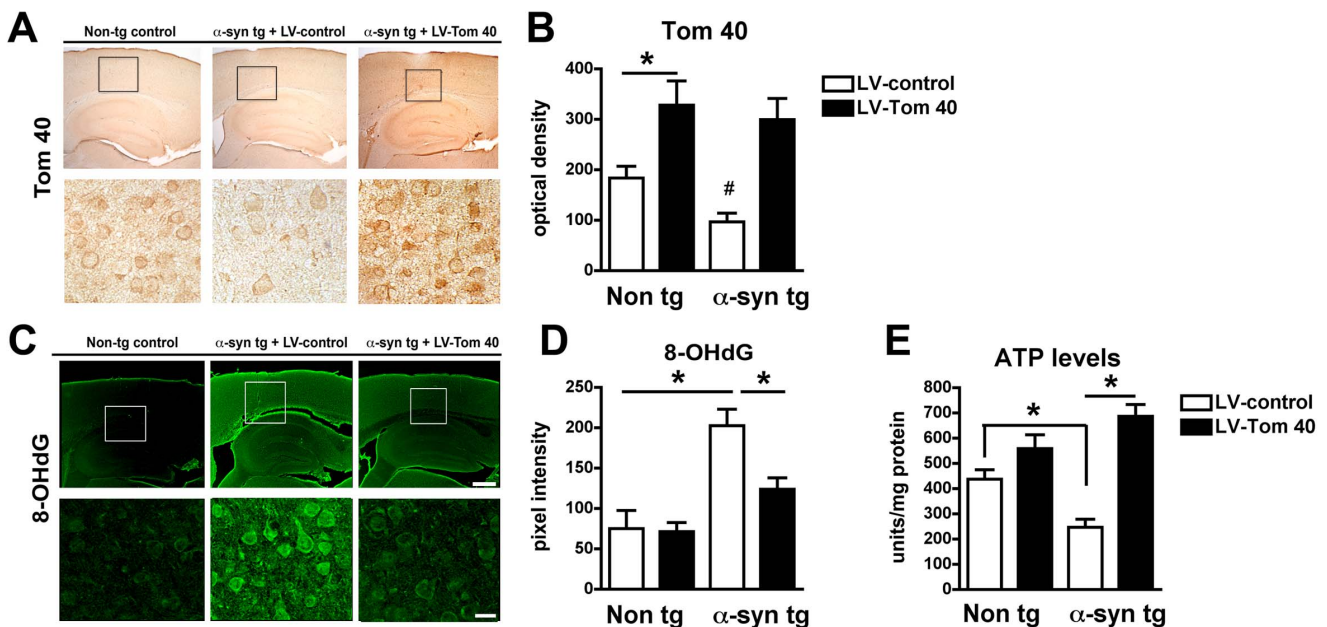


Figure 5. Lentiviral delivery of Tom40 ameliorates α -Syn-induced mitochondrial alterations in transgenic mice. (A) Tom40 immunostaining in sagittal brain sections at the level of the hippocampus of non-transgenic (non-tg) controls and α -Syn-tg mice injected with control lentivirus (LV-control) or with lentiviral delivery of Tom40. (B) Stereological analysis of Tom40 immunoreactivity (optical density) showing increased signal after LV-Tom40 treatment. (C) Fluorescent immunolabeling of oxidative DNA lesions (anti-8-OHdG) in control and α -Syn tg mice treated with LV-control or LV-Tom40. Scale bar represents 100 μ m in the upper panel and 15 μ m in the lower panel. (D) Stereological analysis of anti-8-OHdG staining intensity reveals normal oxidative lesion load in tg animals overexpressing Tom40. (E) Quantification of ATP levels on brain homogenates from mice treated with LV-control or LV-Tom40. * p <0.05 per Student's t test. doi:10.1371/journal.pone.0062277.g005

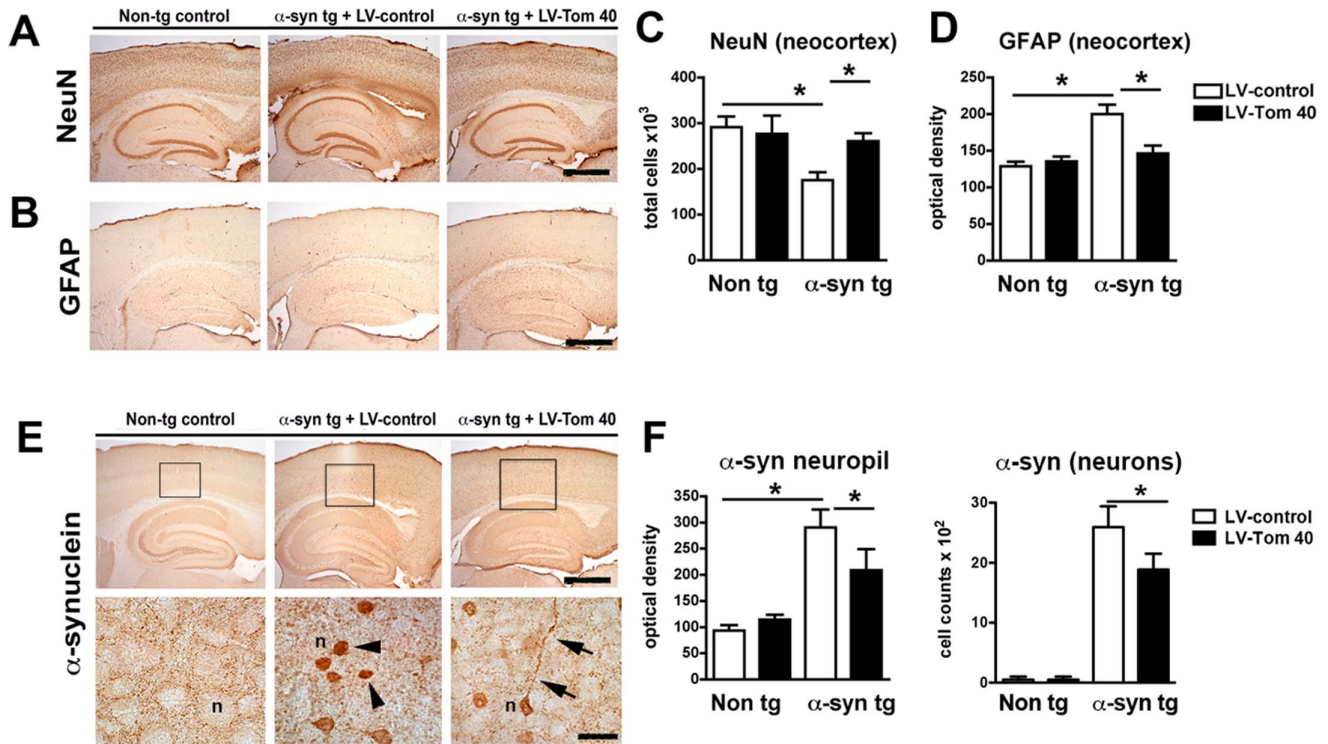


Figure 6. Tom40 overexpression reduces accumulation of α -Syn in neuronal cells. (A and B) Immunostaining of neuronal (anti-Neu N, upper panel) and glial (anti-GFAP, lower panel) cells in non-transgenic control animals and α -Syn tg mice that received LV-control or LV-Tom40 gene therapy. Scale bar represents 250 μ m. (C and D) Stereological analysis of anti-NeuN and anti-GFAP-labeling, respectively (number of positive cells). (E) Effect of Tom40 overexpression on α -Syn levels: immunostaining of α -Syn on sagittal brain sections from non-transgenic (non-tg) controls, α -Syn-tg mice that received control lentivirus and α -Syn-tg mice injected with Tom40-lentivirus. Scale bar represents 250 μ m in the upper panel and 50 μ m in the lower panel. (F) Stereological analysis of α -Syn immunosignal (number of positive cells) showing reduced α -Syn levels on Tom40-overexpressing animals. * p <0.05 per Student's *t* test. doi:10.1371/journal.pone.0062277.g006

mitochondrial content, increased ROS production, increased DNA oxidative damage and reduced ATP production. The effects of A30P mutant on mitochondria were subtle and its accumulation did not alter Tom40 levels. While the precise molecular mechanism that mediates the differential effects of α -Syn isoforms on Tom40 and mitochondrial effects need to be further investigated, it has been reported that wild type and A30P differentially interact with vesicles mimicking the outer mitochondrial membrane, and while both proteins have no affinity for it, increased temperature of incubation increases the binding of the wild-type, but not the A30P variant [42]. This lower affinity for the outer membrane might alter the interaction between Tom40 and A30P.

Overactivation of autophagy has been suggested as a link between intracellular accumulation of α -Syn and mitochondrial dysfunction. In particular, activation of macroautophagy in primary cortical neurons that overexpress mutant A53T α -Syn leads to massive mitochondrial destruction and loss, associated with bioenergetic deficit and neuronal degeneration [33]. Our results showing reductions in mitochondrial content in wild type and A53T α -Syn-accumulating cells are in line with this report, and mitochondrial loss due to increased autophagy is a plausible explanation.

Mice overexpressing mutant A53T α -Syn develop mitochondrial degeneration and apoptotic-like death of neocortical, brainstem, and motor neurons [15]. We show here that overexpression of wt α -Syn is also associated with mitochondrial dysfunction. Extensive deletions on mtDNA should in principle

impact the expression of mitochondrial genome-encoded genes, including subunits of complexes I-IV. We provide evidence showing altered expression of complex I members, in line with robust findings in human PD [4].

Dopaminergic neurons at the SN are the most severely affected cell type in PD. Recent post mortem single cell analyses in brains of sporadic PD patients and controls have shown that these neurons contain high levels of clonally expanded somatic mtDNA deletions [6,43]. This deletion load, higher than 50% of total mtDNA in the neurons of PD patients is sufficient to cause mitochondrial dysfunction [6]. We show that overexpression of human wt α -Syn in mice is also associated with the accumulation of such somatic and clonally expanded mtDNA deletions. Furthermore, we provide evidence at the single neuron resolution, supporting the notion that α -Syn accumulation correlates with higher frequency of mtDNA deletions. Consistent with mtDNA damage and complex I deficiency, α -Syn accumulation also lead to decreased ATP production, indicative of mitochondrial impairment.

Our data show a strong association between accumulation of α -Syn, and impaired energy production, increased oxidative stress and mtDNA damage and aberrant mitochondrial import. As many of these factors can be either a cause or a consequence of the others, it is difficult to establish a clear sequence of events. For example, α -Syn is known to interfere with complex I itself, but, on the other hand, complex I inhibition promotes the aggregation of α -Syn [44]. It is plausible that many of these events feed a vicious cycle that contributes to disease progression. We observed that

wild type and A53T α -Syn accumulation triggered mitochondrial dysfunction via decays in Tom40. These alterations are highly relevant for the pathology of both, autosomal dominant and sporadic PD manifestations.

A potential protective role of Tom40 preventing protein misfolding and aggregation might not be exclusive for α -Syn. Several genetic wide association studies have linked TOM40 polymorphisms with age of onset distribution in late-onset Alzheimer's disease (AD) manifestations (for a review see [45]). TOM40 is in linkage disequilibrium with APOE ϵ 4 allele, an established risk factor for AD [46]. Importantly, accumulation of neurofilament light proteins (NFL) in the cerebrospinal fluid (CSF) which marks neuronal damage, is higher in AD patients that carry the APOE ϵ 4 allele, but only if they do not carry the short poly-T variant of TOM40 associated with late onset AD, suggesting that this mitochondrial protein may be protective against the dose effect of APOE ϵ 4 [47].

Apo-E isoforms differentially regulate amyloid β (A β) aggregation and clearance in the brain, and have distinct functions in regulating brain lipid transport, glucose metabolism, neuronal signaling, neuroinflammation, and mitochondrial function [48]. The protective function of TOM40 in AD might be related to A β aggregation and mitochondrial function integrity, in a similar fashion as we report here for α -Syn in PD. In support of this notion, TOM40 is significantly down-regulated in blood [49] and frontal cortex of AD subjects compared to controls and is related to abnormal mitochondrial dynamics in the brain [50]. Strikingly, truncated amyloid precursor protein (APP) accumulates in the protein import channels of mitochondria in AD brains, forming stable complexes with TOM40 causing mitochondrial dysfunction. Moreover, accumulation of APP in mitochondria varies with severity of AD [51].

The protective effects of TOM40 might also extend beyond α -Syn and A β , as a recent study reported decreased levels of

TOM40 in the striatum and frontal cortex of Huntington's disease (HD) patients, associated with increased oxidative stress and mutant Huntingtin oligomer accumulation in nuclei and mitochondria [52]. Although the functional relationship between TOM40 decay and mitochondrial alterations remains to be clarified for AD and HD, TOM40 appears as a novel pathological theme common to "misfolding protein diseases" supporting our hypothesis that decreased Tom40 leads to mitochondrial dysfunction and oxidative burden that feed a cycle of protein misfolding and neuronal damage.

Our results show that lentivirus-mediated delivery of Tom40 was able to ameliorate the molecular phenotype observed in α -Syn tg mice, as ATP production was restored and oxidative damage was reduced in Tom40-treated animals, supporting a role for Tom40 as a target for α -Syn-mediated mitochondrial dysfunction. Importantly, we found that Tom40 gene therapy resulted in reduced neurodegeneration and inflammation in the brains of α -Syn tg mice, and effect that might contribute with decreased accumulation of α -Syn in neuronal cells as we observed in that treatment group. We hypothesized that higher levels of Tom40 results in improved mitochondrial function, reducing oxidative loads and possibly facilitating α -Syn degradation by autophagy or preventing its misfolding [53,54].

In sum, we provide evidence showing that the interaction of α -Syn with the mitochondrial protein import machinery, in particular Tom40, might be an upstream event in α -Syn-induced neurotoxicity and that targeting of this protein might represent a novel therapeutic strategy in PD.

Author Contributions

Conceived and designed the experiments: AB PD BS EM. Performed the experiments: AB BS PD ER AA ME CL SM AK MM EP TK. Analyzed the data: AB PD BS EM. Wrote the paper: AB PD BS EM.

References

- Schapiro AH (2008) Mitochondria in the aetiology and pathogenesis of Parkinson's disease. *Lancet Neurol* 7: 97–109.
- Banerjee R, Starkov AA, Beal MF, Thomas B (2009) Mitochondrial dysfunction in the limelight of Parkinson's disease pathogenesis. *Biochim Biophys Acta* 1792: 651–663.
- Mann VM, Cooper JM, Daniel SE, Srai K, Jenner P, et al. (1994) Complex I, iron, and ferritin in Parkinson's disease substantia nigra. *Ann Neurol* 36: 876–881.
- Schapiro AH, Cooper JM, Dexter D, Jenner P, Clark JB, et al. (1989) Mitochondrial complex I deficiency in Parkinson's disease. *Lancet* 1: 1269.
- Bindoff LA, Birch-Machin M, Cartledge NE, Parker WD Jr, Turnbull DM (1989) Mitochondrial function in Parkinson's disease. *Lancet* 2: 49.
- Bender A, Krishnan KJ, Morris CM, Taylor GA, Reeve AK, et al. (2006) High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. *Nat Genet* 38: 515–517.
- Kravtsov Y, Kudryavtseva E, McKee AC, Geula C, Kowall NW, et al. (2006) Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons. *Nat Genet* 38: 518–520.
- Iwai A, Masliah E, Yoshimoto M, Ge N, Flanagan L, et al. (1995) The precursor protein of non-A beta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system. *Neuron* 14: 467–475.
- Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, et al. (1997) Alpha-synuclein in Lewy bodies. *Nature* 388: 839–840.
- Hashimoto M, Masliah E (1999) Alpha-synuclein in Lewy body disease and Alzheimer's disease. *Brain Pathol* 9: 707–720.
- Trojanowski JQ, Lee VM (2000) "Fatal attractions" of proteins. A comprehensive hypothetical mechanism underlying Alzheimer's disease and other neurodegenerative disorders. *Ann N Y Acad Sci* 924: 62–67.
- Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, et al. (2003) alpha-Synuclein locus triplication causes Parkinson's disease. *Science* 302: 841.
- Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, et al. (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 276: 2045–2047.
- Masliah E, Rockenstein E, Veinbergs I, Mallory M, Hashimoto M, et al. (2000) Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. *Science* 287: 1265–1269.
- Martin LJ, Pan Y, Price AC, Sterling W, Copeland NG, et al. (2006) Parkinson's disease alpha-synuclein transgenic mice develop neuronal mitochondrial degeneration and cell death. *J Neurosci* 26: 41–50.
- Song DD, Shults CW, Sisk A, Rockenstein E, Masliah E (2004) Enhanced substantia nigra mitochondrial pathology in human alpha-synuclein transgenic mice after treatment with MPTP. *Exp Neurol* 186: 158–172.
- Parihar MS, Parihar A, Fujita M, Hashimoto M, Ghafourifar P (2008) Mitochondrial association of alpha-synuclein causes oxidative stress. *Cell Mol Life Sci*.
- Li WW, Yang R, Guo JC, Ren HM, Zha XL, et al. (2007) Localization of alpha-synuclein to mitochondria within midbrain of mice. *Neuroreport* 18: 1543–1546.
- Parihar MS, Parihar A, Fujita M, Hashimoto M, Ghafourifar P (2009) Alpha-synuclein overexpression and aggregation exacerbates impairment of mitochondrial functions by augmenting oxidative stress in human neuroblastoma cells. *Int J Biochem Cell Biol* 41: 2015–2024.
- Devi L, Raghavendran V, Prabhu BM, Avadhani NG, Anandatheerthavarada HK (2008) Mitochondrial import and accumulation of alpha-synuclein impairs complex I in human dopaminergic neuronal cultures and Parkinson's disease brain. *J Biol Chem*.
- Dauer W, Kholodilov N, Vila M, Trillat AC, Goodchild R, et al. (2002) Resistance of alpha-synuclein null mice to the parkinsonian neurotoxin MPTP. *Proc Natl Acad Sci U S A* 99: 14524–14529.
- Klivenyi P, Siwek D, Gardian G, Yang L, Starkov A, et al. (2006) Mice lacking alpha-synuclein are resistant to mitochondrial toxins. *Neurobiol Dis* 21: 541–548.
- Reeve AK, Krishnan KJ, Elson JL, Morris CM, Bender A, et al. (2008) Nature of mitochondrial DNA deletions in substantia nigra neurons. *Am J Hum Genet* 82: 228–235.
- Krishnan KJ, Bender A, Taylor RW, Turnbull DM (2007) A multiplex real-time PCR method to detect and quantify mitochondrial DNA deletions in individual cells. *Anal Biochem* 370: 127–129.
- He L, Chinnery PF, Durham SE, Blakely EL, Wardell TM, et al. (2002) Detection and quantification of mitochondrial DNA deletions in individual cells by real-time PCR. *Nucleic Acids Res* 30: e68.
- Naldini L, Verma IM (2000) Lentiviral vectors. *Adv Virus Res* 55: 599–609.

27. Marr RA, Rockenstein E, Mukherjee A, Kindy MS, Hersh LB, et al. (2003) Nprilysin gene transfer reduces human amyloid pathology in transgenic mice. *J Neurosci* 23: 1992–1996.
28. Mucke L, Abraham CR, Ruppe MD, Rockenstein EM, Toggas SM, et al. (1995) Protection against HIV-1 gp120-induced brain damage by neuronal expression of human amyloid precursor protein. *J Exp Med* 181: 1551–1556.
29. Toggas SM, Masliah E, Rockenstein EM, Rall GF, Abraham CR, et al. (1994) Central nervous system damage produced by expression of the HIV-1 coat protein gp120 in transgenic mice. *Nature* 367: 188–193.
30. McFarland MA, Ellis CE, Markey SP, Nussbaum RL (2008) Proteomics analysis identifies phosphorylation-dependent alpha-synuclein protein interactions. *Mol Cell Proteomics* 7: 2123–2137.
31. Yamamoto H, Itoh N, Kawano S, Yatsukawa Y, Momose T, et al. (2011) Dual role of the receptor Tom20 in specificity and efficiency of protein import into mitochondria. *Proceedings of the National Academy of Sciences of the United States of America* 108: 91–96.
32. Kruger R, Kuhn W, Muller T, Woitalla D, Graeber M, et al. (1998) Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nature genetics* 18: 106–108.
33. Choubey V, Safulina D, Vaarmann A, Cagalinec M, Wareski P, et al. (2011) Mutant A53T alpha-synuclein induces neuronal death by increasing mitochondrial autophagy. *The Journal of biological chemistry* 286: 10814–10824.
34. Beal MF (2009) Therapeutic approaches to mitochondrial dysfunction in Parkinson's disease. *Parkinsonism & related disorders* 15 Suppl 3: S189–194.
35. Clayton DA (1984) Transcription of the mammalian mitochondrial genome. *Annual review of biochemistry* 53: 573–594.
36. Totterdell S, Meredith GE (2005) Localization of alpha-synuclein to identified fibers and synapses in the normal mouse brain. *Neuroscience* 135: 907–913.
37. Devi L, Anandatheerthavarada HK (2010) Mitochondrial trafficking of APP and alpha synuclein: Relevance to mitochondrial dysfunction in Alzheimer's and Parkinson's diseases. *Biochim Biophys Acta* 1802: 11–19.
38. Zhang L, Zhang C, Zhu Y, Cai Q, Chan P, et al. (2008) Semi-quantitative analysis of alpha-synuclein in subcellular pools of rat brain neurons: An immunogold electron microscopic study using a C-terminal specific monoclonal antibody. *Brain Res*.
39. Meisinger C, Ryan MT, Hill K, Model K, Lim JH, et al. (2001) Protein import channel of the outer mitochondrial membrane: a highly stable Tom40-Tom22 core structure differentially interacts with preproteins, small tom proteins, and import receptors. *Mol Cell Biol* 21: 2337–2348.
40. Pennington K, Peng J, Hung CC, Banks RE, Robinson PA (2010) Differential effects of wild-type and A53T mutant isoform of alpha-synuclein on the mitochondrial proteome of differentiated SH-SY5Y cells. *J Proteome Res* 9: 2390–2401.
41. Goncalves S, Outeiro TF (2013) Assessing the Subcellular Dynamics of Alpha-synuclein Using Photoactivation Microscopy. *Molecular neurobiology*.
42. Zigonceanu IG, Yang YJ, Krois AS, Haque E, Pielak GJ (2012) Interaction of alpha-synuclein with vesicles that mimic mitochondrial membranes. *Biochimica et biophysica acta* 1818: 512–519.
43. Bender A, Schwarzkopf RM, McMillan A, Krishnan KJ, Rieder G, et al. (2008) Dopaminergic midbrain neurons are the prime target for mitochondrial DNA deletions. *J Neurol*.
44. Fornai F, Schluter OM, Lenzi P, Gesi M, Ruffoli R, et al. (2005) Parkinson-like syndrome induced by continuous MPTP infusion: convergent roles of the ubiquitin-proteasome system and alpha-synuclein. *Proc Natl Acad Sci U S A* 102: 3413–3418.
45. Roses AD (2010) An inherited variable poly-T repeat genotype in TOMM40 in Alzheimer disease. *Archives of neurology* 67: 536–541.
46. Roses AD, Lutz MW, Amrine-Madsen H, Saunders AM, Crenshaw DG, et al. (2009) A TOMM40 variable-length polymorphism predicts the age of late-onset Alzheimer's disease. *Pharmacogenomics J*.
47. Bruno D, Pomara N, Nierenberg J, Ritchie JC, Lutz MW, et al. (2012) Levels of cerebrospinal fluid neurofilament light protein in healthy elderly vary as a function of TOMM40 variants. *Experimental gerontology* 47: 347–352.
48. Liu CC, Kanekiyo T, Xu H, Bu G (2013) Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. *Nature reviews Neurology*.
49. Chong MS, Goh LK, Lim WS, Chan M, Tay L, et al. (2013) Gene Expression Profiling of Peripheral Blood Leukocytes Shows Consistent Longitudinal Downregulation of TOMM40 and Upregulation of KIR2DL5A, PLOD1, and SLC2A8 Among Fast Progressors in Early Alzheimer's Disease. *Journal of Alzheimer's disease : JAD* 34: 399–405.
50. Manczak M, Calkins MJ, Reddy PH (2011) Impaired mitochondrial dynamics and abnormal interaction of amyloid beta with mitochondrial protein Drp1 in neurons from patients with Alzheimer's disease: implications for neuronal damage. *Human molecular genetics* 20: 2495–2509.
51. Devi L, Prabhu BM, Galati DF, Avadhani NG, Anandatheerthavarada HK (2006) Accumulation of amyloid precursor protein in the mitochondrial import channels of human Alzheimer's disease brain is associated with mitochondrial dysfunction. *The Journal of neuroscience: the official journal of the Society for Neuroscience* 26: 9057–9068.
52. Shirendeb U, Reddy AP, Manczak M, Calkins MJ, Mao P, et al. (2011) Abnormal mitochondrial dynamics, mitochondrial loss and mutant huntingtin oligomers in Huntington's disease: implications for selective neuronal damage. *Human molecular genetics* 20: 1438–1455.
53. Scherz-Shouval R, Elazar Z (2007) ROS, mitochondria and the regulation of autophagy. *Trends in cell biology* 17: 422–427.
54. Spencer B, Potkar R, Trejo M, Rockenstein E, Patrick C, et al. (2009) Beclin 1 gene transfer activates autophagy and ameliorates the neurodegenerative pathology in alpha-synuclein models of Parkinson's and Lewy body diseases. *The Journal of neuroscience: the official journal of the Society for Neuroscience* 29: 13578–13588.