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Pesticide Exposure Exacerbates α-Synucleinopathy in an A53T Transgenic Mouse Model

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The factors initiating or contributing to the pathogenesis of Parkinson's disease and related neurodegenerative synucleinopathies are still largely unclear, but environmental factors such as pesticides have been implicated. In this study, A53T mutant human α-synuclein transgenic mice (M83), which develop α-synuclein neuropathology, were treated with the pesticides paraquat and maneb (either singly or together), and their effects were analyzed. Immunohistochemical and biochemical analyses showed that chronic treatment of M83 transgenic mice with both pesticides (but not with either pesticide alone) drastically increased neuronal α-synuclein pathology throughout the central nervous system including the hippocampus, cerebellum, and sensory and auditory cortices. α-Synuclein-associated mitochondrial degeneration was observed in M83 but not in wild-type α-synuclein transgenic mice. Because α-synuclein inclusions accumulated in pesticide-exposed M83 transgenic mice without a motor phenotype, we conclude that α-synuclein aggregate formation precedes disease onset. These studies support the notion that environmental factors causing nitrative damage are closely linked to mechanisms underlying the formation of α-synuclein pathologies and the onset of Parkinson's-like neurodegeneration. ***(Am J Pathol 2007, 170:658–666; DOI: 10.2353/ajpath.2007.060359)***

Neuronal synucleinopathies are exemplified by Parkinson's disease (PD), the most common neurodegenerative movement disorder, which is characterized pathologically by filamentous α-synuclein (α-syn) inclusions and neuron loss. α-Syn is an abundant, soluble protein primarily expressed at synaptic terminals. Although the exact functions of α-syn are unknown, evidence suggests a role in synaptic activities (reviewed in Ref. 1), including the ability to act as a molecular chaperone that assists in refolding proteins during the process of synaptic release.[2] α-Syn became an intense focus of research when it was identified as the building block of the pathological hallmarks of PD known as Lewy bodies and Lewy neurites.[3,4]

The mechanisms that convert the normally soluble α-syn into insoluble filamentous aggregates in Lewy bodies and Lewy neurites of PD are unknown, but environmental factors have been implicated (reviewed in Refs. 5, 6). For example, pesticides may contribute to the risk for PD[7–13] through oxidative and nitrative mechanisms by generating reactive oxygen species, alkylating reduced thiols, and inhibiting mitochondrial complex I (reviewed in Refs. 5, 6, 14). Of particular importance in this regard are paraquat (PQ) and maneb (MB). PQ resembles the metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) known as MPP^+ because PQ is preferentially toxic to dopaminergic neurons. PQ exposure produces nigral degeneration, behavioral deficits, synaptic alterations, and decreased dopamine levels,[15–19] while it also accelerates the fibrillization of recombinant α-syn *in vitro*.[18,20] Furthermore, MB decreases motor activity and increases the neurotoxicity of MPTP,[21,22] possibly by way of mitochondrial[23] and/or proteasomal inhibition.[24] The toxic effects of PQ and MB are synergistic in mice exposed to both pesticides.[25–27]

To gain insights into the role of PQ and MB in nitrative and oxidative damage and the onset of α-syn pathology versus the development of a motor and other clinical phenotypes, we studied the effects of PQ and MB treatment on trans-

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genic mice that overexpress human A53T α-syn and model PD-like neurodegenerative synucleinopathies.[28]

Materials and Methods

Animals

Tg mice expressing wild-type (WT; M7 line) or A53T (M83 line) human α-syn protein under the control of the prion protein promoter were generated and characterized as described previously.[28] In these studies, homozygous M7 Tg and M83 Tg mouse lines were used in addition to non-Tg (NTg) mice with the same background.

Biochemical Extraction and Protein Assessment

The cerebellum, brain stem, hippocampus, and cortex of euthanized NTg, M7 Tg, and M83 Tg mice were thoroughly homogenized in high salt (HS) buffer (50 mmol/L Tris, pH 7.5, 750 mmol/L NaCl, 5 mmol/L ethylenediamine tetraacetic acid, and protease inhibitor cocktail) and centrifuged at 100,000 × *g* for 25 minutes. Pellets were re-extracted in 1% Triton X-100 and recentrifuged, and myelin was removed by homogenization in 1 mol/L sucrose in HS buffer. After recentrifugation, pellets were extracted in radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 5 mmol/L ethylenediamine tetraacetic acid, 1% NP40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). After another sedimentation step, pellets were sonicated in 70% formic acid. After centrifugation at 100,000 × *g* for 25 minutes, formic acid was removed by lyophilization, and the dried material was resuspended in sodium dodecyl sulfate sample buffer by sonicating and heating the sample to 100°C for 5 minutes. The bicinchoninic acid assay (Pierce, Rockford, IL) was used to determine protein concentrations, and 10 μg of total protein from HS and RIPA fractions and 5 μl of formic acid fractions were loaded on separate lanes of 15% polyacrylamide gels. The expression and distribution of human α-syn, as well as nitrated/oxidized proteins, were analyzed by Western blot analysis. In addition, differences in levels of human α-syn were determined by Western blots using ^{125}I-labeled anti-mouse secondary antibody. The membranes were dried and exposed to a PhosphorImager plate (GE Healthcare, Piscataway, NJ), and the radioactive signal was quantified using ImageQuant software (GE Healthcare).

PQ and MB Treatment of Mice

NTg, M7 Tg, and M83 Tg mice at 3, 8, or 12 months of age were injected intraperitoneally with saline, PQ, MB, or PQ and MB (referred to as PQ/MB hereafter) twice a week for 3 consecutive weeks. PQ was used at a dose of 10 mg/kg in mice of 8 or 12 months of age and a dose of 5 mg/kg in mice of 3 months of age. MB was used at a dose of 30 mg/kg in 8- or 12-month-old mice or a dose of 15 mg/kg in 3-month-old mice. Mice were monitored for behavioral changes and weight loss and then sacrificed by intraperitoneal xylazine and ketamine administration, followed by perfusion with phosphate-buffered saline. Brains were fixed in 70% ethanol/150 mmol/L NaCl for histochemical analysis or frozen for biochemical analysis; many brains were hemi-sectioned for both histochemical and biochemical analyses.

Antibodies

LB509 is a mouse monoclonal antibody (mAb) specific for human α-syn,[29] and Syn303 is a mouse mAb raised against oxidized human α-syn that specifically labels pathological α-syn inclusions.[30] N808 and NSyn14 are mouse mAbs raised against nitrated α-syn; N808 recognizes all nitrated proteins (B.I. Giasson, unpublished data), whereas NSyn14 specifically recognizes nitrated α-syn.[31] The neurofilament (NF) antibody is a polyclonal rabbit antibody.[32] UB1B4 (D. Sampuathu, unpublished data) and UB1510 (Chemicon International, Inc., Temecula, CA) are mouse mAbs that recognize ubiquitin protein. HSP90 is a mouse mAb from Stressen Bioreagents (Ann Arbor, MI). Other antibodies include Lamp-2 (ABL-93; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and cytochrome *c* (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunohistochemistry

Mice were perfused with saline and subsequently with 70% ethanol/150 mmol/L NaCl. Brains were removed and postfixed for approximately 24 hours and then separated into four ~3-mm-thick sections. Brain sections then were processed and dehydrated through a series of graded ethanol solutions to xylene at room temperature, infiltrated with paraffin at 60°C, and cut into 6-μm-thick sections.[33] Immunohistochemistry was performed as described previously[34] using the avidin-biotin complex kit (Vector Laboratories, Burlingame, CA). Double fluorescent immunohistochemistry with Syn303 and NF antibodies was performed as described previously.[31] Staining was analyzed with an Olympus BX51 microscope (Olympus, Tokyo, Japan).

Conventional and Immuno-Electron Microscopy

NTg, M7 Tg, and M83 Tg mice with saline or PB/MB treatment were fixed with 2% paraformaldehyde and 1.5% glutaraldehyde in 0.1 mol/L phosphate-buffered saline (pH 7.4) for conventional EM. Brains were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 mol/L phosphate-buffered saline for immuno-electron microscopy (immuno-EM). Brains were fixed overnight and then cut into 50-μm-thick sections using a Vibratome 3000 (Vibratome, St. Louis, MO). Immuno-EM sections were processed with Syn303, Lamp-2, or cytochrome *c* or for silver enhancement as described previously.[35] Subsequently, sections processed for immunostaining and for conventional EM were dehydrated by a graded series of ethanol, post-

fixed with 1% osmium tetra-oxide, and embedded in EPON812. Ultra-thin sections were cut, stained with uranium acetate, and examined under an electron microscope (JEM-1010; JEOL, Tokyo, Japan).

Results

Pesticide Treatment of Presymptomatic M83 A53T α-Syn Mice Exacerbates α-Syn Pathology

Cohorts of 3-, 8-, and 12-month-old NTg, M7 Tg, and nonsymptomatic M83 Tg mice were exposed to saline, PQ, MB, or PQ/MB. NTg ($n = 5$) and M7 Tg ($n = 14$) mice did not present a phenotype or α-syn neuropathology in any brain regions examined when treated with PQ and/or MB at any age, and they were similar to saline-treated mice in all respects (NTg, $n = 5$; M7 Tg, $n = 5$) (Figure 1, E, K, P, and U for NTg and D, J, O, and T for M7 Tg). Furthermore, no noticeable cell loss in the substantia nigra or other brain regions was observed in NTg, M7 Tg, or M83 Tg mouse lines at any age after PQ and/or MB treatment. Three-month-old M83 Tg mice did not develop α-syn inclusions from chronic pesticide exposure ($n = 6$); however, the majority of the 8- and 12-month-old asymptomatic M83 Tg mice ($n = 7$ of 9 treated) exposed to PQ/MB developed α-syn-positive inclusions of varying sizes in the granule layer of the cerebellar cortex, molecular layer of hippocampus, and deep layers of the somatosensory and auditory cortices (Figure 1, A, B, F, G, L, M, Q, and R). However, these mice did not show inclusion formation after treatment with either PQ or MB alone or with saline (Figure 1, C, H, N, and S). Thus, PQ/MB exposure in M83 Tg mice induced α-syn neuropathology in brain regions not observed in these mice as reported previously.[28] α-Syn aggregates were also found in the brain stem, including the raphe nucleus, arcuate nucleus, striatum, and thalamus in seven of the nine treated M83 Tg mice (Figure 1, F and G; data not shown). Although untreated M83 Tg mice eventually develop α-syn inclusions in the brain stem, striatum, and thalamus,[28] the proportion of affected mice is much higher than expected at this age. The α-syn-positive inclusions noted here, including cell body and punctated structures, were essentially colocalized with NF staining (Figure 1, V, W, and X). Interestingly, however, the small punctated inclusions in the hippocampus were not localized with NF (Figure 1, Y, Z, and ZA), NeuN, or glial fibrillary acidic protein (data not shown), and none of the α-syn-positive inclusions were Thioflavin-S positive. In addition, the saline-treated, asymptomatic M83 Tg mice at 8 and 12 months of age did not accumulate α-syn in any brain region, including the brain stem (Figure 1, C, H, N, and S). These results suggest that PQ/MB exposure induced α-syn pathology in these brain areas.

Most M83 Tg mice treated with both PQ/MB had extensive brain pathology, although this was not associated with a motor phenotype. PQ/MB exposure often produces a motor phenotype in mice, most likely due to the extensive loss of nigral neurons.[15–19] The mice presented in this study did not undergo nigral degeneration from pesticide treatment, which may explain the lack of a motor phenotype in these mice. However, these mice did present with inclusions in novel/rare brain regions, possibly due to the abnormal overexpression of human mutant α-syn in these brain regions. Treated M83 Tg mice displayed only minor weight loss, hind limb weakness, and diminished spontaneous movements but no paralysis, tremors, or hunched posture (data not shown). Therefore, α-syn pathology precedes phenotype and symptom onset in PQ/MB-treated mice.

Pesticide Exposure Leads to Disorganized and Degenerated Mitochondria and Accumulation of Lysosomes

EM studies were performed on brain sections collected from NTg, M7 Tg, and M83 Tg mice exposed to either saline or PQ/MB ($n = 1$ to 3 mice per group). In both brain stem and hippocampal sections of saline-treated NTg, M7 Tg, and M83 Tg mice, the majority of mitochondria had healthy appearances (data not shown). In contrast, PQ/MB-treated mice easily revealed membrane structure disorganization (Figure 2, A–D). In particular, samples from treated M83 Tg mice showed membranous lamination (Figure 2D), whereas treated NTg and M7 Tg mice failed to do so (Figure 2C). Immuno-EM observations using Syn303 did not show α-syn-positive immunolabeling associated with normal mitochondria from NTg, M7 Tg, or M83 Tg mouse brains. Positive α-syn labeling was only present in mitochondria with disrupted membranes (arrowheads in Figure 2, E and G), and this observation was unique to PQ/MB-treated M83 Tg mice.

The majority of neurons in PQ/MB-treated mice contained more lysosomes in neurons than that of saline-treated mice. Lysosomes frequently had a degenerated membrane/tubular appearance and/or associated with lipid particles (Figure 2, A, B, C, F, H, and I). The amount of lysosome induction was higher in treated Tg mice than in treated NTg mice, and membrane-like components within the lysosomes resembled damaged mitochondria as reported recently.[36] Anti-Lamp-2 antibody was used in immuno-EM studies to identify lysosomes and identified various lysosomal structures (Supplemental Figure 1A, *http://ajp.amjpathol.org*). Immuno-EM revealed that these components in the lysosomes frequently associated with α-syn (Figure 2H). Furthermore, degraded membrane-like components in the lysosomes were labeled for cytochrome *c* in both PQ/MB-treated M7 and M83 Tg mice (Figure 2H). The mitochondria and lysosomes associated with α-syn immunoreactivity may have contributed to the enhancement of immunoreactivity in PQ/MB-treated M83 Tg mice that were noted in the light microscopy level. These results suggest that PQ/MB treatment leads to mitochondrial damage and lysosomal degradation in all mice used in this study, yet there was significantly more mitochondrial damage in treated M83 Tg mice than M7 Tg or NTg mice.

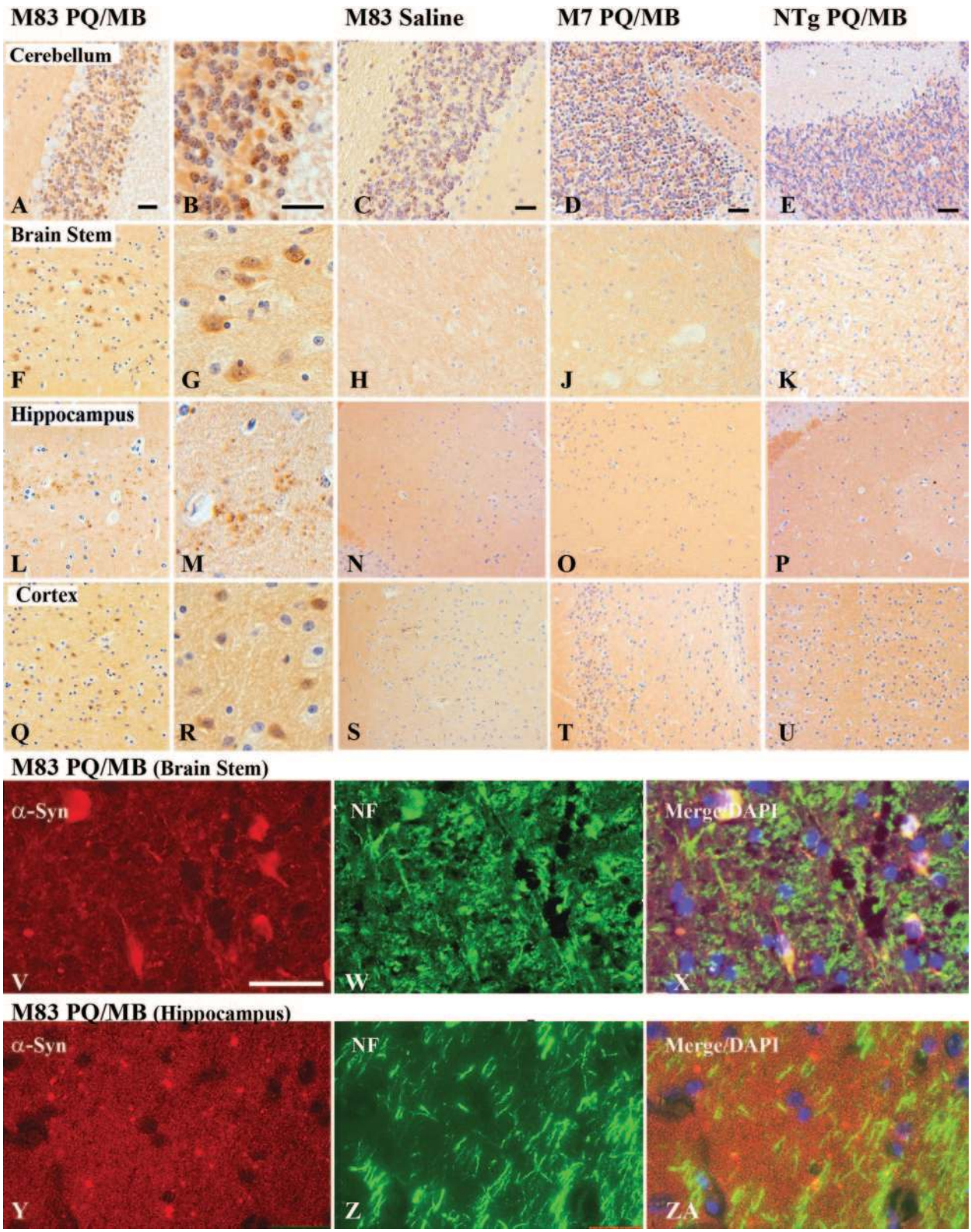

Figure 1. Comparison of human α-syn in brains of NTg, M7 Tg, and M83 Tg mice. Micrographs of cerebellum, brain stem, hippocampus, and cortex of saline- or PQ/MB-treated mice using Syn303 anti-α-syn antibody demonstrate the presence of human α-syn inclusions and/or accumulations in the cerebellum, brain stem, hippocampus, and sensory and auditory cortices of PQ/MB-treated M83 Tg mice at 8 months of age at low power (**A**, **F**, **L**, and **Q**) and at high power (**B**, **G**, **M**, and **R**). Inclusions do not appear in these brain regions of 8-month-old saline-treated M83 Tg mice (**C**, **H**, **N**, and **S**). PQ/MB-treated M7 Tg (**D**, **J**, **O**, and **T**) and NTg (**E**, **K**, **P**, and **U**) mice do not develop similar α-syn pathologies in these or other regions. LB509 anti-α-syn antibody showed staining patterns similar to Syn303. α-Syn and NF double fluorescent immunohistochemistry revealed α-syn localization in neuronal elements exemplified by the raphe nucleus in the brain stem of M83 Tg mouse after PQ/MB treatment (**V**, **W**, and **X**). Interestingly, however, in the same group of mice, the majority of punctated α-syn labeling did not colocalize with NF immunoreactivity (**Y**, **Z**, and **ZA**) or with glial fibrillary acidic protein immunoreactivity (data not shown). Scale bar = 50 μm.

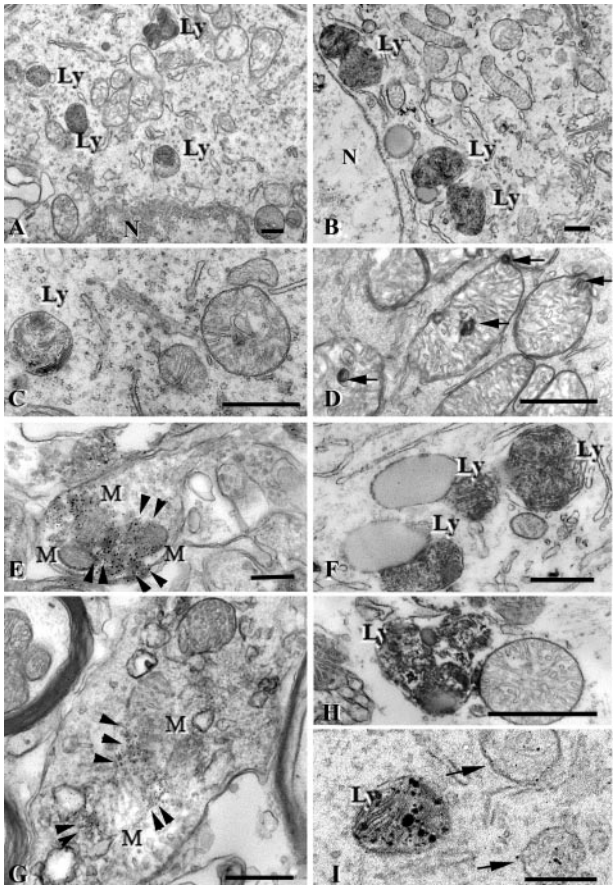

Figure 2. EM analysis of PQ/MB-induced mitochondrial damage and lysosomes. EM analysis shows that PQ/MB exposure to NTg (data not shown), M7 Tg (**A**), and M83 Tg (**B**) mice leads to herniation of mitochondria and damage to their membranes in addition to the accumulation of lysosomes. High-power magnification reveals disorganized mitochondrial crystae in PQ/MB-exposed M7 Tg (**C**) and M83 Tg (**D**) mice. Mitochondrial membrane laminations (**arrow** in **D**) are noted only in treated M83 Tg mice. Furthermore, the damaged mitochondria from these mice associate with Syn303 α-syn immunolabeling (**E**, **G**; arrowheads), whereas intact mitochondria are not associated with α-syn immunolabeling. Lysosomes containing tubules and membrane-like structures, which appear to be degraded mitochondria (**C**, **F**, **H**, and **I**), are frequently found in all PQ/MB-treated mice (**C** and **F**). Undefined components are immunohistochemically labeled by Syn303 anti-α-syn antibody (**H**), whereas its membrane-like components are frequently labeled by cytochrome *c* within these lysosomes (**I**). **Arrows** in **I** indicate mitochondria labeled by cytochrome *c*. Ly, lysosome; M, severely damaged mitochondria; N, nucleus. **A**, **B**, and **G** are from the hippocampus; **C**, **D**, **E**, and **F** are from the brain stem. Scale bars = 50 nm.

Exposure to PQ/MB Promotes the Formation of Filamentous α-Syn Aggregates in Axon Terminals

Additional immuno-EM studies were performed using the Syn303 antibody on brain stem and hippocampal sections collected from NTg, M7 Tg, and M83 Tg mice exposed to either saline or PQ/MB. Among the mice used in the current study, PQ/MB-treated mice were the only group of mice that showed exacerbated α-syn pathology. Plastic-embedded semithin sections of hippocampi from saline-treated M83 Tg mice indicated the presence of modest α-syn-positive immunolabeling that was closely associated with neuronal cell bodies

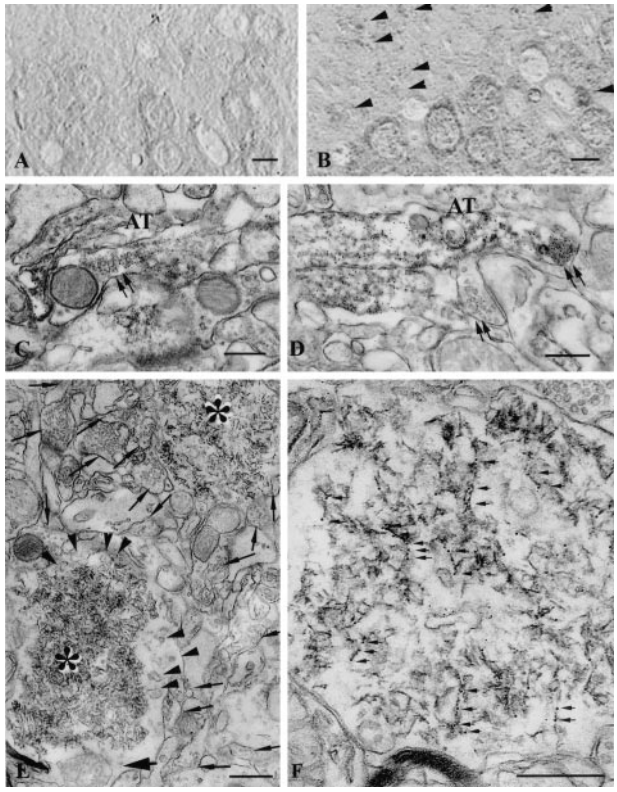

Figure 3. EM analysis of PQ/MB-induced filamentous α-syn inclusions. In plastic embedded sections, saline-treated M83 Tg mice show α-syn-positive labeling closely associated with neuronal cell bodies in the hippocampus (**A**), whereas their PQ/MB-treated M83 Tg mouse counterparts indicate enhanced α-syn immunoreactivity and more punctated inclusions (**arrowhead** in **B**). Saline-treated (**C**) and PQ/MB-treated (**D**) mice show synaptic vesicles (**double arrows**) and filaments in axonal terminals (AT), both of which were α-syn positive. PQ/MB-treated M83 Tg mice contain clusters of α-syn filaments (**white asterisk**) in varying densities (**E** and **F**). Filamentous aggregates are located in the cytoplasm within a membranous boundary. Vesicles of various sizes (**arrowheads**) and mitochondria (**large arrow**) are located within this boundary, and a number of synaptic terminals (**elongated arrow**) are noticeable at the outer limb. Individual filaments labeled by α-syn immunoreactivity are noticeable at higher magnification (**small arrows** in **F**). Ly, lysosomes; M, mitochondria; AT, axon terminal. Scale bars: 10 μm (**A** and **B**); 50 nm (**C**–**F**).

(Figure 3A), whereas their PQ/MB-treated counterparts showed an enhanced staining and a greater number of punctated inclusions with α-syn-positive immunoreactivity (Figure 3B). Ultrastructural analysis of these sections confirmed that the saline- and PQ/MB-treated M83 Tg mice had positive α-syn labeling surrounding synaptic vesicles at axon terminals (Figure 3, C and D). These positively labeled terminals were found widely in the neurophil, including the area adjacent to neuronal cell bodies.

Interestingly, immuno-EM for α-syn revealed clusters of filamentous aggregates composed of fibrils with a diameter of approximately 10 to 20 nm (Figure 3, E and F). The range of diameter in its individual filament was comparable with that of α-syn filaments in our previous study.[37] The degree of filament density and the size of aggregates (100 to 500 nm) varied greatly. These distinct filaments were decorated with α-syn-positive silver grains (arrow in Figure 3F). The α-syn-positive filamentous aggregates were more abundant in sections from the hip-

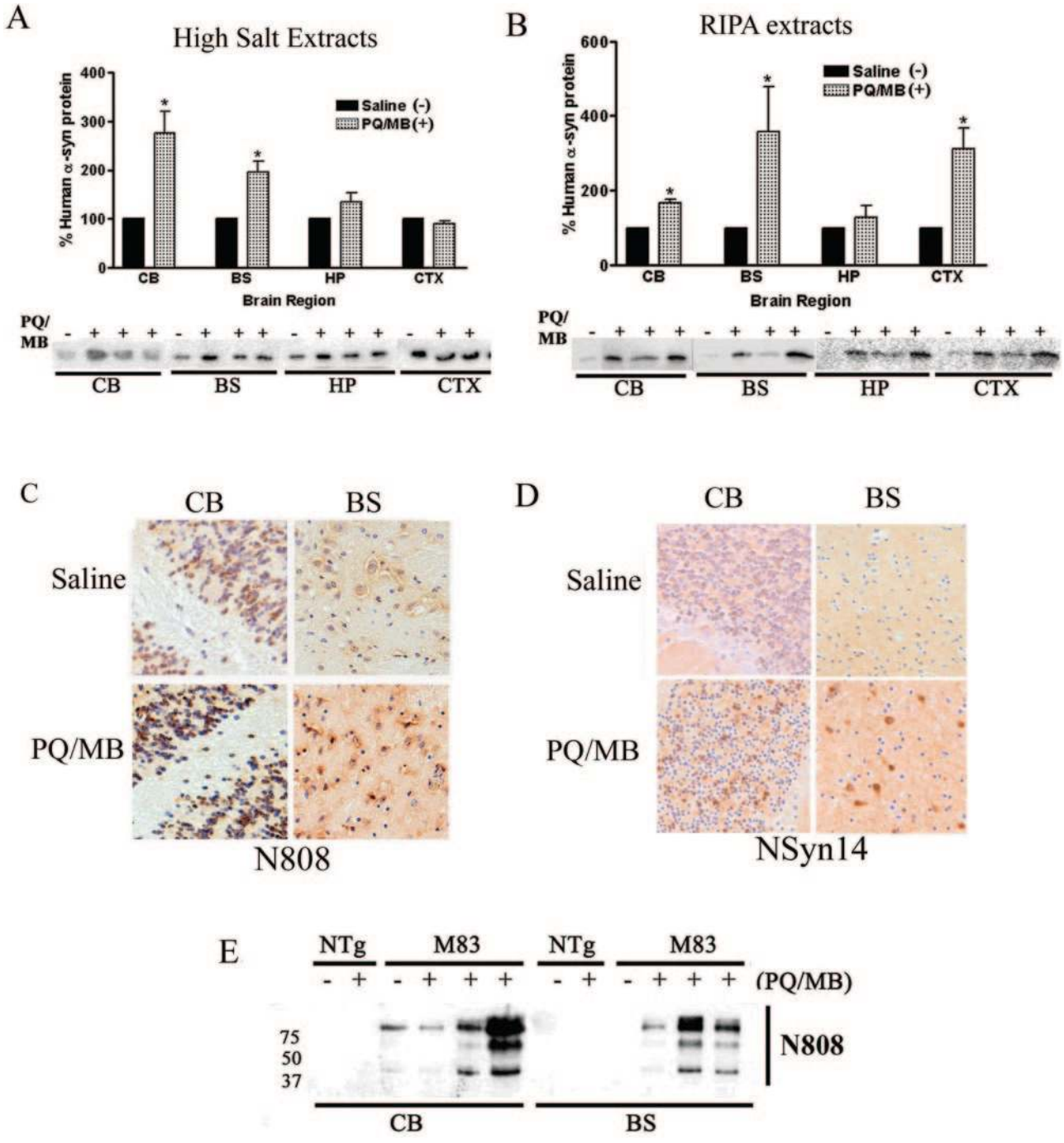

Figure 4. PQ/MB treatment increases α-syn levels in different brain regions of M83 Tg mice. Levels of human α-syn protein were quantified using LB509 anti-α-syn primary antibody, ^{125}I-labeled anti-mouse secondary antibody, and ImageQuant software after M83 Tg mice were treated with saline (−) or PQ/MB (+), and brain regions were sequentially extracted as described in Materials and Methods. Analysis of HS fractions (**A**) from cerebellum (CB), brain stem (BS), hippocampus (HP), and cortex (CTX) showed a significant increase in α-syn levels after PQ/MB treatment in the CB and BS regions, whereas analysis of RIPA-soluble proteins (**B**) identified significant differences in the amount of human α-syn in CB, BS, and CTX after PQ/MB treatment. Representative blots probed with ^{125}I-labeled anti-mouse secondary antibody and quantified with ImageQuant software are shown in **A** and **B**. Immunohistochemistry (**C**) and Western blot (**E**) analyses show an increase in protein nitration in PQ/MB-treated M83 Tg mice compared with untreated M83 Tg mice, demonstrated by anti-N808 antibody. Immunohistochemical (**D**) analyses using NSyn14 antibody indicate that the up-regulation in nitration (shown in **C** and **E**) is partially due to the specific nitration of α-syn caused by PQ/MB exposure in M83 Tg mice.

pocampus than those from the raphe nucleus. These filamentous aggregates were clearly located within a membrane boundary. Various sizes of vesicles and mitochondria were located in proximity to each other (Figure 3E). At the outer limbs of the membrane-bound structure, a number of presynaptic terminals were detected. Judging from the location and structure, it was presumed that these aggregates were located in neuronal processes. NTg and M7 Tg mice with or without PQ/MB treatment failed to demonstrate such filamentous structures.

Effects of Pesticide Exposure on the Levels and Solubility of Human α-Syn

Quantitative biochemical analysis of human α-syn expression in the cerebellum, brain stem, hippocampus, and cortex of treated and untreated M83 Tg mice ($n = 3$ control and $n = 6$ treated mice per group) showed a dramatic increase in levels of HS-soluble α-syn in the cerebellum and brain stem (Figure 4A). Furthermore, quantification of extracts from these same brain regions of treated and untreated M83 Tg mice showed a significant increase in RIPA-soluble α-syn protein levels in the cerebellum, brain stem, and cortex (Figure 4B). In addition, levels of nitrated proteins increased significantly in HS fractions obtained from the cerebellum and brain stem from PQ/MB-treated M83 Tg mice (Figure 4, C and E), and the nitrated α-syn-specific antibody NSyn14 used in immunohistochemistry showed that these inclusions were composed of nitrated α-syn protein (Figure 4D), thereby providing support for nitrative damage due to chronic pesticide exposure. Finally, biochemical and histochemical comparisons of treated versus nontreated mice showed no significant changes in the levels of HSP90 (Supplemental Figure 1, B and C) and ubiquitin (Supplemental Figure 1, D and E) in pesticide-exposed mice.

Discussion

Although the mechanisms underlying PD are not entirely understood, it has been established that α-syn, mitochondrial dysfunction, and nitrative/oxidative damage play significant roles in this process (reviewed in Refs. 5, 6). Mitochondrial impairments have been found in human PD cases because there is a 30 to 40% decrease in complex I activity and immunoreactivity in the substantia nigra.[38–42] Mitochondrial deficits may lead to the production of reactive oxygen species and hence oxidative stress, which has also been linked to PD pathogenesis (reviewed in Refs. 5, 43).

Several recently published studies have shown that treatment with pesticides and other complex I inhibitors can mimic PD pathology in some animal models. For example, chronic administration of MPTP can lead to nigral degeneration, behavioral impairments, and proteinaceous inclusions that are immunoreactive for α-syn and ubiquitin in WT mouse brains, whereas mice lacking α-syn do not undergo behavioral deficits or neuronal degeneration under the same conditions.[44] Other studies also showed that the pesticide rotenone, which is a classical inhibitor of mitochondrial complex I, can lead to nigral degeneration associated with the formation of α-syn-positive inclusions.[45–48] Finally, intraperitoneal injections of PQ into NTg and Tg mice expressing human WT and A53T α-syn under the control of the tyrosine hydroxylase promoter resulted in an up-regulation of α-syn expression and the formation of α-syn-positive inclusions; however, nigral degeneration induced by PQ was only found in NTg mice, suggesting that human α-syn expression in these Tg mice may play a protective role against pesticide-induced damage.[15,18] Other investigators have shown that overexpression of α-syn in mice leads to alterations in mitochondria, increased free radical production, and neuronal death.[49] Taken together, these and other studies suggest a link between oxidative/nitrative stress and α-syn aggregation in PD pathogenesis. To investigate these mechanisms further, transgenic mice expressing human WT or A53T α-syn under the control of the prion protein promoter were challenged with PQ and MB.

Previous studies demonstrated that M83 A53T human α-syn Tg mice, but not M7 WT α-syn Tg mice, recapitulate key features of a PD-like neuronal synucleinopathy.[28] Here, we showed that exposure to PQ and MB augmented the formation of neuronal α-syn pathologies in M83, but not M7 Tg or NTg mice, throughout the CNS, including regions of the brain stem, hippocampus, cerebellum, cortex, thalamus, and striatum. Similar to previous studies,[15] these pesticide-treated mice show elevated levels of α-syn in the brain stem and do not undergo nigral degeneration, suggesting a potential protective role for α-syn in this brain region. The accumulation of α-syn in the HS-insoluble/RIPA-soluble biochemical fractions reflects the increased formation of inclusions in the respective brain regions. However, biochemical analysis of mouse brains also revealed an up-regulation of aqueous-soluble α-syn protein in the cerebellum and brain stem of PQ/MB-treated mice. Although the mechanism of this stress-induced α-syn up-regulation remains to be elucidated, it may be linked to the impairment of α-syn degradation by lysosomes and autophagosomes.[50] PQ/MB exposure also resulted in significant increases in the levels of nitrated proteins. Therefore, the formation of α-syn-positive aggregates due to PQ/MB exposure could be a combined result of increased levels of α-syn and nitrative and oxidative challenges. However, the formation of aggregates in the cortex without increased levels of RIPA-soluble α-syn indicates that nitrative stress may be a more important factor. These results suggest that the A53T mutation in α-syn confers susceptibility to nitrative damage, which can then lead to increased α-syn accumulation and aggregate formation. This finding is consistent with the effect of the A53T mutation in increasing the propensity of α-syn to fibrillize.[37,51,52]

In summary, these studies strengthen the link between pesticide exposure and PD and support the concept that nitrative damage plays a role in mechanisms underlying the formation of α-syn pathologies and the onset of PD-like neurodegeneration. In addition, genetic mutations, as exemplified by the A53T mutation in α-syn, as well as environmental toxins can synergistically lead to neurodegenerative synucleinopathies. Genetic mutations in the α-syn gene may prime neurons to develop pathology, but a secondary insult such as an environmental toxin may catalyze or enhance this process by damaging mitochondria and/or reducing the amount of α-syn degradation. Finally, the experimental model described here recapitulates some of the features of PD and provides important insights into disease mechanism.

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References

1. Norris EH, Giasson BI, Lee VMY: Alpha-synuclein: normal function and role in neurodegenerative diseases. Curr Top Dev Biol 2004, 60:17–54
2. Chandra S, Gallardo G, Fernandez-Chacon R, Schluter OM, Sudhof TC: Alpha-synuclein cooperates with CSPalpha in preventing neurodegeneration. Cell 2005, 123:383–396
3. Forman MS, Lee VMY, Trojanowski JQ: Nosology of Parkinson's disease: looking for the way out of a quagmire. Neuron 2005, 47:479–482
4. Spillantini MG, Schmidt ML, Lee VMY, Trojanowski JQ, Jakes R, Goedert M: Alpha-synuclein in Lewy bodies. Nature 1997, 388: 839–840
5. Beal MF: Mitochondria, oxidative damage, and inflammation in Parkinson's disease. Ann NY Acad Sci 2003, 991:120–131
6. Moore DJ, West AB, Dawson VL, Dawson TM: Molecular pathophysiology of Parkinson's disease. Annu Rev Neurosci 2005, 28:57–87
7. Barbeau PA, Cloutier T, Plasse L, Paris S: Environmental and genetic factors in the etiology of Parkinson's disease. Adv Neurol 1986, 45:299–306
8. Barbeau A, Roy M, Bernier G, Campanella G, Paris S: Ecogenetics of Parkinson's disease: prevalence and environmental aspects in rural areas. Can J Neurol Sci 1987, 14:36–41
9. Hertzman C, Weins M, Bowering D, Snow B, Calne D: Parkinson's disease: a case-control study of occupational and environmental risk factors. Am J Ind Med 1990, 17:349–355
10. Jimenez-Jimenez FJ, Mateo D, Gimenez-Roldman S: Exposure to well water and pesticides in Parkinson's disease: a case-control study in the Madrid area. Mov Disord 1992, 7:149–152
11. Tanner CM, Chen B, Wang W, Peng M, Liu Z, Liang X, Kao LC, Gilley DW, Goetz CG, Schoenberg BS: Environmental factors and Parkinson's disease: a case-control study in China. Neurology 1989, 39:660–664
12. Ferraz HB, Bertolucci PH, Pereira JS, Lima JG, Andrade LA: Chronic exposure to the fungicide maneb may produce symptoms and signs of CNS manganese intoxication. Neurology 1988, 38:550–553
13. Meco G, Bonifati V, Vanacore N, Fabrizio E: Parkinsonism after chronic exposure to the fungicide maneb (manganese ethylene-bis-dithiocarbamate). Scand J Work Environ Health 1994, 20:301–305
14. Ischiropoulos H, Beckman JS: Oxidative stress and nitration in neurodegeneration: cause, effect, or association? J Clin Invest 2003, 111:163–169
15. Manning-Bog AB, McCormack AL, Purisai MG, Bolin LM, Di Monte DA: Alpha-synuclein overexpression protects against paraquat-induced neurodegeneration. J Neurosci 2003, 23:3095–3099
16. Fredriksson A, Fredriksson M, Eriksson P: Neonatal exposure to paraquat or MPTP induces permanent changes in striatum dopamine and behavior in adult mice. Toxicol Appl Pharmacol 1993, 122:258–264
17. Liou HH, Chen RC, Tsai YF, Chen WP, Chang YC, Tsai MC: Effects of paraquat on the substantia nigra of the Wistar rats: neurochemical, histological, and behavioral studies. Toxicol Appl Pharmacol 1996, 137:34–41
18. Manning-Bog AB, McCormack AL, Li J, Uversky VN, Fink AL, Di Monte DA: The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: paraquat and alpha-synuclein. J Biol Chem 2002, 277:1641–1644
19. McCormack AL, Thiruchelvam M, Manning-Bog AB, Thiffault C, Langston JW, Cory-Slechta DA, Di Monte DA: Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. Neurobiol Dis 2002, 10:119–127
20. Uversky VN, Li J, Fink AL: Pesticides directly accelerate the rate of alpha-synuclein fibril formation: a possible factor in Parkinson's disease. FEBS Lett 2001, 500:105–108
21. Takahashi RN, Rogerio R, Zanin M: Maneb enhances MPTP neurotoxicity in mice. Res Commun Chem Pathol Pharmacol 1989, 66:167–170
22. Morato GS, Lemos T, Takahashi RN: Acute exposure to maneb alters some behavioral functions in the mouse. Neurotoxicol Teratol 1989, 11:421–425
23. Zhang J, Fitsanakis VA, Gu G, Jing D, Ao M, Amarnath V, Montine TJ: Manganese ethylene-bis-dithiocarbamate and selective dopaminergic neurodegeneration in rat: a link through mitochondrial dysfunction. J Neurochem 2003, 84:336–346
24. Zhou Y, Shie FS, Piccardo P, Montine TJ, Zhang J: Proteasomal inhibition induced by manganese ethylene-bis-dithiocarbamate: relevance to Parkinson's disease. Neuroscience 2004, 128:281–291
25. Thiruchelvam M, Richfield EK, Baggs RB, Tank AW, Cory-Slechta DA: The nigrostriatal dopaminergic system as a preferential target of repeated exposures to combined paraquat and maneb: implications for Parkinson's disease. J Neurosci 2000, 20:9207–9214
26. Thiruchelvam M, Richfield EK, Goodman BM, Baggs RB, Cory-Slechta DA: Developmental exposure to the pesticides paraquat and maneb and the Parkinson's disease phenotype. Neurotoxicology 2002, 23:621–633
27. Thiruchelvam M, McCormack A, Richfield EK, Baggs RB, Tank AW, Di Monte DA, Cory-Slechta DA: Age-related irreversible progressive nigrostriatal dopaminergic neurotoxicity in the paraquat and maneb model of the Parkinson's disease phenotype. Eur J Neurosci 2003, 18:589–600
28. Giasson BI, Duda JE, Quinn SM, Zhang B, Trojanowski JQ, Lee VMY: Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. Neuron 2002, 34:521–533
29. Baba M, Nakajo S, Tu PH, Tomita T, Nakaya K, Lee VMY, Trojanowski JQ, Iwatsubo T: Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. Am J Pathol 1998, 152:879–884
30. Duda JE, Giasson BI, Mabon ME, Lee VMY, Trojanowski JQ: Novel antibodies to synuclein show abundant striatal pathology in Lewy body diseases. Ann Neurol 2002, 52:205–210
31. Uryu K, Giasson BI, Longhi L, Martinez D, Murray I, Conte V, Nakamura M, Saatman K, Talbot K, Horiguchi T, McIntosh T, Lee VMY, Trojanowski JQ: Age-dependent synuclein pathology following traumatic brain injury in mice. Exp Neurol 2003, 184:214–224
32. Schmidt ML, Carden MJ, Lee VMY, Trojanowski JQ: Phosphate dependent and independent neurofilament epitopes in the axonal swellings of patients with motor neuron disease and controls. Lab Invest 1987, 56:282–294
33. Trojanowski JQ, Schuck T, Schmidt ML, Lee VMY: Distribution of tau proteins in the normal human central and peripheral nervous system. J Histochem Cytochem 1989, 37:209–215
34. Duda JE, Giasson BI, Gur TL, Montine TJ, Robertson D, Biaggioni I, Hurtig HI, Stern MB, Gollomp SM, Grossman M, Lee VMY, Trojanowski JQ: Immunohistochemical and biochemical studies demonstrate a distinct profile of alpha-synuclein permutations in multiple system atrophy. J Neuropathol Exp Neurol 2000, 59:830–841
35. Galvin JE, Uryu K, Lee VMY, Trojanowski JQ: Axon pathology in Parkinson's disease and Lewy body dementia hippocampus contains alpha-, beta-, and gamma-synuclein. Proc Natl Acad Sci USA 1999, 96:13450–13455
36. Yazdani U, German DC, Liang CL, Manzino L, Sonsalla PK, Zeevalk GD: Rat model of Parkinson's disease: chronic central delivery of 1-methyl-4-phenylpyridinium (MPP(+)). Exp Neurol 2006, 200: 172–183
37. Giasson BI, Uryu K, Trojanowski JQ, Lee VMY: Mutant and wild type human alpha-synucleins assemble into elongated filaments with distinct morphologies in vitro. J Biol Chem 1999, 274:7619–7622
38. Hattori N, Tanaka M, Ozawa T, Mizuno Y: Immunohistochemical studies on complexes I, II, III, and IV of mitochondria in Parkinson's disease. Ann Neurol 1991, 30:563–571
39. Janetzky B, Hauck S, Youdim MB, Riederer P, Jellinger K, Pantucek F, Zochling R, Boissl KW, Reichmann H: Unaltered aconitase activity, but decreased complex I activity in substantia nigra pars compacta

of patients with Parkinson's disease. Neurosci Lett 1994, 169: 126–128
40. Mann VM, Cooper JM, Krige D, Daniel SE, Schapira AH, Marsden CD: Brain, skeletal muscle and platelet homogenate mitochondrial function in Parkinson's disease. Brain 1992, 115:333–342
41. Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD: Mitochondrial complex I deficiency in Parkinson's disease. J Neurochem 1990, 54:823–827
42. Bindoff LA, Birch-Machin M, Cartlidge NE, Parker Jr WD, Turnbull DM: Mitochondrial function in Parkinson's disease. Lancet 1989, 2:49
43. Norris EH, Giasson BI: Role of oxidative damage in protein aggregation associated with Parkinson's disease and related disorders. Antioxid Redox Signal 2005, 7:672–684
44. Fornai F, Schluter OM, Lenzi P, Gesi M, Ruffoli R, Ferrucci M, Lazzeri G, Busceti CL, Pontarelli F, Battaglia G, Pellegrini A, Nicoletti F, Ruggieri S, Paparelli A, Sudhof TC: Parkinson-like syndrome induced by continuous MPTP infusion: convergent roles of the ubiquitin-proteasome system and alpha-synuclein. Proc Natl Acad Sci USA 2005, 102:3413–3418
45. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT: Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 2000, 3:1301–1306
46. Betarbet R, Canet-Aviles RM, Sherer TB, Mastroberardino PG, McLendon C, Kim JH, Lund S, Na HM, Taylor G, Bence NF, Kopito R, Seo BB, Yagi T, Yagi A, Klinefelter G, Cookson MR, Greenamyre JT: Intersecting pathways to neurodegeneration in Parkinson's disease: effects of the pesticide rotenone on DJ-1, alpha-synuclein, and the ubiquitin-proteasome system. Neurobiol Dis 2006, 22:404–420
47. Sherer TB, Kim JH, Betarbet R, Greenamyre JT: Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and alpha-synuclein aggregation. Exp Neurol 2003, 179:9–16
48. Sherer TB, Betarbet R, Testa CM, Seo BB, Richardson JR, Kim JH, Miller GW, Yagi T, Matsuno-Yagi A, Greenamyre JT: Mechanism of toxicity in rotenone models of Parkinson's disease. J Neurosci 2003, 23:10756–10764
49. Hsu LJ, Sagara Y, Arroyo A, Rockenstein E, Sisk A, Mallory M, Wong J, Takenouchi T, Hashimoto M, Masliah E: Alpha-synuclein promotes mitochondrial deficit and oxidative stress. Am J Pathol 2000, 157:401–410
50. Cuervo AM, Stefanis L, Fredenburg R, Lansbury PT, Sulzer D: Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. Science 2004, 305:1292–1295
51. Conway KA, Harper JD, Lansbury PT: Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease. Nat Med 1998, 4:1318–1320
52. Serpell LC, Berriman J, Jakes R, Goedert M, Crowther RA: Fiber diffraction of synthetic alpha-synuclein filaments shows amyloid-like cross-beta conformation. Proc Natl Acad Sci USA 2000, 97: 4897–4902