ELSEVIER

Neurobiology of Disease

www.elsevier.com/locate/ynbdi
Neurobiology of Disease 21 (2006) 541 – 548

Mice lacking alpha-synuclein are resistant to mitochondrial toxins

Peter Klivenyi,[a] Donald Siwek,[b] Gabrielle Gardian,[a] Lichuan Yang,[a] Anatoly Starkov,[a] Carine Cleren,[a] Robert J. Ferrante, [b] Neil W. Kowall,[b] Asa Abeliovich,[c] and M. Flint Beal [a,*]

[a]*Department of Neurology and Neuroscience, Weill Medical College of Cornell University, New York-Presbyterian Hospital, 525 East 68th Street, New York, NY 10021, USA*
[b]*Geriatric Research Education and Clinical Center, Bedford VA Medical Center, Bedford, MA and Neurology, Pathology, and Psychiatry Departments, Boston University School of Medicine, Boston, MA 02118, USA*
[c]*Department of Neurology, College of Physicians and Surgeons, Columbia University, New York -Presbyterian Hospital, New York, NY 10021, USA*

Received 25 May 2005; revised 12 August 2005; accepted 28 August 2005
Available online 18 November 2005

Abnormalities in the function of α-synuclein are implicated in the pathogenesis of Parkinson's disease (PD). We found that α-synuclein-deficient mice are resistant to MPTP-induced degeneration of dopaminergic neurons. There was dose-dependent protection against loss of both dopamine in the striatum and dopamine transporter (DAT) immunoreactive neurons in the substantia nigra. These effects were not due to alterations in MPTP processing. We found that α-synuclein-deficient mice are also resistant to both malonate and 3-nitropropionic acid (3-NP) neurotoxicity. There was reduced generation of reactive oxygen species in α-synuclein-deficient mice following administration of 3-NP. These findings implicate α-synuclein as a modulator of oxidative damage, which has been implicated in neuronal death produced by MPTP and other mitochondrial toxins.

Keywords: MPTP; Paraquat; 3-Nitropropionic acid; Malonate; MPP^+; MPP^+ levels; Vesicular uptake; DHBA

Introduction

A role of α-synuclein in the pathophysiology of Parkinson's disease (PD) has been under intense investigation following the finding that mutations in α-synuclein are associated with dominantly inherited PD, and that α-synuclein appears to be the most abundant protein in Lewy bodies (Polymeropoulos et al., 1997; Spillantini et al., 1998; Kruger et al., 1999). α-Synuclein is also part of glial cytoplasmic inclusions of multiple system atrophy (Tu et al., 1998). It is associated with the neuronal intranuclear inclusions of Huntington's disease (Furlong et al., 2000; Mezey et al., 2000). Overexpression of both wild-type and mutated α-synuclein produces neurotoxicity in *Drosophila*, mice, and rats (Feany and Bender, 2000; Kirik et al., 2002; Richfield et al., 2002; Giasson et al., 2002; Lee et al., 2001a).

The normal physiologic role of α-synuclein is unknown. α-Synuclein is widely expressed in the nervous system, where it is found in presynaptic nerve terminals closely associated with presynaptic vesicles (Goedert, 2001; Cole and Murphy, 2002). Immunoelectron microscopy, as well as cell-fractionation studies, suggest that α-synuclein is not stably associated with synaptic membranes (Clayton and George, 1999; Kahle et al., 2000). α-Synuclein, however, undergoes a marked conformational change upon binding to cellular membranes and interacts with a number of vesicle-related and microtubule-associated molecules (Goedert, 2001). In the substantia nigra dopaminergic neurons, α-synuclein may regulate the rate of refilling of the releasable pool of synaptic vesicles (Abeliovich et al., 2000).

The neurotoxicity of α-synuclein may be related to its fibrillization. Both PD α-synuclein mutations [Ala^{53}-Thr (A53T) and [Ala^{36}-Pro (A30P)] accelerate the formation of nonfibrillar oligomeric protofibrils in vitro, but A30P inhibits the conversion of protofibrils to fibrils (Conway et al., 2000). Dopamine is oxidatively linked to α-synuclein, and this prevents the protofibril-to-fibril conversion, causing accumulation of α-synuclein protofibrils (Conway et al., 2001). Toxicity in *Drosophila* is dependent on phosphorylation of serine 129 (Chen and Feany, 2005).

Oxidative damage can cross-link α-synuclein with the formation of dityrosine, and α-synuclein can be nitrated (Souza et al., 2000; Paxinou et al., 2001). Lewy bodies are nitrated in PD, suggesting that peroxynitrite mediated oxidative damage may contribute to disease pathogenesis (Giasson et al., 2000). α-Synuclein increases oxidative damage in vitro and sensitizes cells to oxidative insults (Hsu et al., 2000; Ko et al., 2000; Ostrerova-Golts et al., 2000; Lee et al., 2001b).

Therefore, we examined whether a deficiency of α-synuclein alters susceptibility to mitochondrial toxins which produce oxidative

* Corresponding author. Fax: +1 212 746 8532.
E-mail address: fbeal@mail.med.cornell.edu (M.F. Beal).
Available online on ScienceDirect (www.sciencedirect.com).

0969-9961/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.nbd.2005.08.018

damage. We examined the effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a toxin which produces an animal model of PD (Beal, 2001). We also examined the susceptibility of α-synuclein-deficient mice to the mitochondrial toxins malonate and 3-NP, which produce striatal toxicity which closely mimic many features of HD (Beal et al., 1993; Brouillet and Hantraye, 1995).

Materials and methods

All experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of experimental animals. The α-synuclein$^{-/-}$ mice were generated as previously described (Abeliovich et al., 2000). Briefly, a gene-targeting construct was generated in which the first two exons, encoding amino acids 1–41 and upstream untranslated sequences were deleted. This targeting sequence was electroporated into embryonic stem (ES) cells to produce α-synuclein$^{-/-}$ mice. The resulting homozygous α-synuclein$^{-/-}$ mice are viable, normal in size, and fertile. The α-synuclein$^{-/-}$ mice have an absence of α-synuclein mRNA and protein. MPTP (Research Biochemicals, Wayland, MA) was dissolved in phosphate-buffered saline (PBS), and 20 mg/kg was injected i.p. in a volume of 0.15 ml every 2 h for three doses in wild-type littermate control, hemizygous α-synuclein$^{+/-}$ mice and homozygous α-synuclein$^{-/-}$ mice. Control mice received PBS vehicle. Eight to ten animals were examined in each group. Mice were sacrificed 7 days after MPTP injections, and the striata were dissected and placed in chilled 0.1 M perchloric acid. Samples were sonicated and centrifuged, and an aliquot was taken for measurements of dopamine, 3, 4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) by HPLC using electrochemical detection as previously described. Concentrations of dopamine and metabolites were expressed as nanograms per milligram of protein (means ± SEM). In a similar experimental paradigm, histopathological evaluation of the effects of MPTP i.p. injections was performed in 6 wild-type littermate control and 6 α-synuclein$^{-/-}$ mice. The mice received the same treatment regimen as above. Seven days after the last MPTP injection, the mice will be deeply anesthetized and transcardially perfused with phosphate-buffered 4% paraformaldehyde.

The brains were postfixed for 24 h and cryoprotected in a graded series of 10 and 20% glycerol/2% DMSO solution. Tissue specimens were subsequently serially cut on a cryostat at 50 μm, stored in six separate collection wells, and stained for Nissl using cresyl violet as previously described. Cut tissue sections of the midbrain were immunostained for dopamine transporter (DAT) (DAT antisera, 1:500 dilution; Chemicon International, Inc). DAT labels neurons of the substantia nigra pars compacta. Midbrain sections through both the left and the right substantia nigra from the bregma levels ñ 3.08 to ñ 3.16 mm and intraaural levels 0.72 to 0.64 mm were analyzed by microscopic videocapture. Stereologic counts of Nissl- and DAT-positive neurons within the substantia nigra pars compact were computed using Neurolucida (Microbrightfield, Colchester, VT) image analysis software. The dissector counting technique was employed, in which all neurons were counted in an unbiased selection of serial sections in a defined volume of the substantia nigra.

Quantification of cross-sectional area and density measurements of DAT immunoreactive striatal terminals were made using a Nikon Optiphot laboratory microscope configures with a "CoolSnap", cooled CCS digital camera Media Cybernetics, Silver Spring, MD). Spatially calibrated digital photomicroscope images of the striatum were taken at 1×, and a sample area (AOI) of 21030 μm^2 was used to measure the integrated optical density of MPTP lesions. Two reading from several sections from each animal were made by measuring the optical density of DAT staining in a striatal lesion and then at a nearby unaffected striatum. To normalize the data, the measurements were expressed as ratio of the unaffected tissue measurement divided by the value of the lesion. These measurements were made in PBS wild-type injected animals and in homozygous knockouts, heterozygous knockouts, and in MPTP injected wild-type animals.

To determine whether MPTP uptake or metabolism was altered, a single dose of MPTP 20 mg/kg was administered i.p., and mice were sacrificed at 90 min after the last injection (n = 6 per group). Striatal tissue was dissected, and samples were sonicated in 0.1 M perchloric acid. MPP^+ levels were quantified by HPLC with UV detection at 295 nm. An aliquot of supernatant was injected onto a Brownlee Aquapore X03-224 cation exchange column (Rainin, Woburn, MA). Samples were eluted isocratically with 90% 0.1 M acetic acid and 75 mM triethylamine HC1, pH 2.3, adjusted with formic acid and 10% acetonitrile.

Malonate (Sigma, St. Louis, MO) was dissolved in PBS and the pH adjusted to 7.4 with HC1. Intrastriatal injections of 1.0 μl containing 1.5 μmol of malonate were made within the left striatum 0.5 mm anterior to bregma, 2.1 mm lateral to the midline, 3.8 mm ventral to the skull surface of both wild-type littermate control and α-synuclein-deficient mice, using a 10-μl Hamilton syringe fitted with a 26-gauge needle (n = 10 for each group). At 7 days, mice were deeply anesthetized and transcardially perfused with 4% buffered paraformaldehyde. Striatal lesion volumes were determined on Nissl-stained sections from the areas of pallor in which there was increased gliosis and neuronal loss. Stereological analysis of lesion volumes using the Cavalieri principle was computed from serial sections through the striata using Neurolucida software (Microbrightfield).

3-Nitropropionic acid (Sigma) was dissolved in PBS (pH adjusted to 7.4) and injected intraperitoneally nine times at 12-h intervals, using a dose of 50 mg/kg per injection in both α-synuclein-deficient and littermate wild-type mice (n = 10 for each group). The animals were deeply anesthetized and transcardially perfused with buffered 4% paraformaldehyde 3–5 h after the last 3-NP injection and processed for histopathology as above. Striatal lesion volumes were determined on Nissl-stained sections from the areas of pallor in which there was increased gliosis and neuronal loss. Stereological analysis of lesion volumes using the Cavalieri principle was computed from serial sections through the striata using Neurolucida software (Microbrightfield).

To study effects of 3-NP on succinate dehydrogenase (SDH) activity, mice were injected i.p. with 3-NP as follows: 1st day, 2 × 25 mg/kg; 2nd and 3rd days, 2 × 50 mg/kg; 4th day, 50 and 25 mg/kg; 5th day, 50 mg/kg; mice were sacrificed 5 h after the last injection We examined 4 mice in each group. Mice were sacrificed by cervical dislocation, and striata and cortex samples were immediately dissected on ice-cold plate, weighted and frozen at −80°C overnight. Frozen tissue was thawed on ice and manually homogenized in 1 ml or 20 mM HEPES (pH 7.6) buffer using a Dounce-type homogenizer. Homogenates were diluted with 20 mM HEPES (pH 7.6) to 1 mg tissue per ml and loaded into a 96-well plate.

SDH activity was measured spectrophotometrically by following 2,6-dichloro-phenolindipohenol (DCPIP) reduction (Arrigon and

Singer, 1962) at 595 nm with a HTS7000^{+} plate reader (Perkin-Elmer, USA). The reaction mixture contained 80 μM DCPIP, 5 mM succinate, 1 μM rotenone, 1 mM KCN, 40 μM coenzyme Q1, and 20 mM HEPES 9 pH 7.6). The baseline reaction rate was obtained by supplementing the reaction mixture with 100 mM thenoyltrifluoroacetone (TTFA, a specific inhibitor or SDH). Specific SDH activity was obtained by subtracting the baseline rate from that in the absence of TTFA. Extinction coefficient for DCPIP was taken as $E_{(600\ nm)} = 21 \times cm^{-1} \times mM^{-1}$.

4HBA/3,4DHBA measurement

4-Hydroxybutyric acid (4HBA) at a dose of 600 mg/kg (dissolved in saline) was administered i.p to wild-type, hemizygous, and homozygous knockout mice ($n = 8$ for each group). After 20 min, 3-NP (100 mg/kg i.p., dissolved in saline) was administered. Twenty minutes following 3-NP administration, a second injection of 4HBA (600 mg/kg, i.p.) was made, and the animals were sacrificed after 20 min. Additional set of animals were sacrificed 20 min after a single 600 mg/kg dose of 4HBA. The brains were rapidly removed, dissected on chilled glass plate, and stored at − 80°C until measurement. Eighty microliters 0.5 M PCA was added to the striatal tissues, sonicated, and centrifuged for 10 min at 10,000 *g*. The supernatant was used to determine the concentrations of 3,4-dihydroxybutyric acid (3,4-DHBA) and 4HBA using HPLC/EC. Samples were separated on a Super ODS 4.6 × 50 mm, 2 mm column (Tosohass, Mongomeryville, PA). The mobile phase was delivered at 1 ml/min (ESA model 480 pump, ESA, Chelmsford, MA) consisting of 100 mM NaH_2PO_4 (pH 2.8 with phosphoric acid), 6.5% methanol (v/v). Analytes were detected using a dual potentiostat electrochemical detector (Coulochem II, ESA) and a dual coulometric electrode analytical cell (model 5011, ESA). The potential applied to the first and the second electrodes were +225 and +700 mV, respectively. 3,4-DHBA was detected on the first electrode, and 4HBA on the second. Data collection was performed using an ESA 500 data station. Under these conditions, the limit of detection for 3,4-DHBA is about 0.5 pg on the column, and the chromatogram was completed in under 10 min. Data were expressed as the mean ± SEM values of the ratio (3,4-DHBA/4-HBA) (nmol/mmol).

Statistical analysis

Results are expressed as the mean ± SEM. Statistical comparisons were made using unpaired student's *t* test or one-way ANOVA followed by Fisher's PLSD post hoc tests.

Results

The effects of administration of MPTP in wild-type controls and α-synuclein knockout mice are shown in Fig. 1. The dose of MPTP we used (3 × 20 mg/kg) produced significant dopamine depletion of 70% in wild-type controls. The same dose of MPTP produced a significant 49% depletion of dopamine in heterozygous knockout mice and 41% in homozygous knockout mice ($P < 0.05$ and $P < 0.002$ respectively). This MPTP dose produced a significant depletion of DOPAC and HVA in controls. There was a trend of increasing levels of the metabolites in the knockout groups, but only the HVA level reached statistical significance in the homozygous knockout mice. The decreased sensitivity to

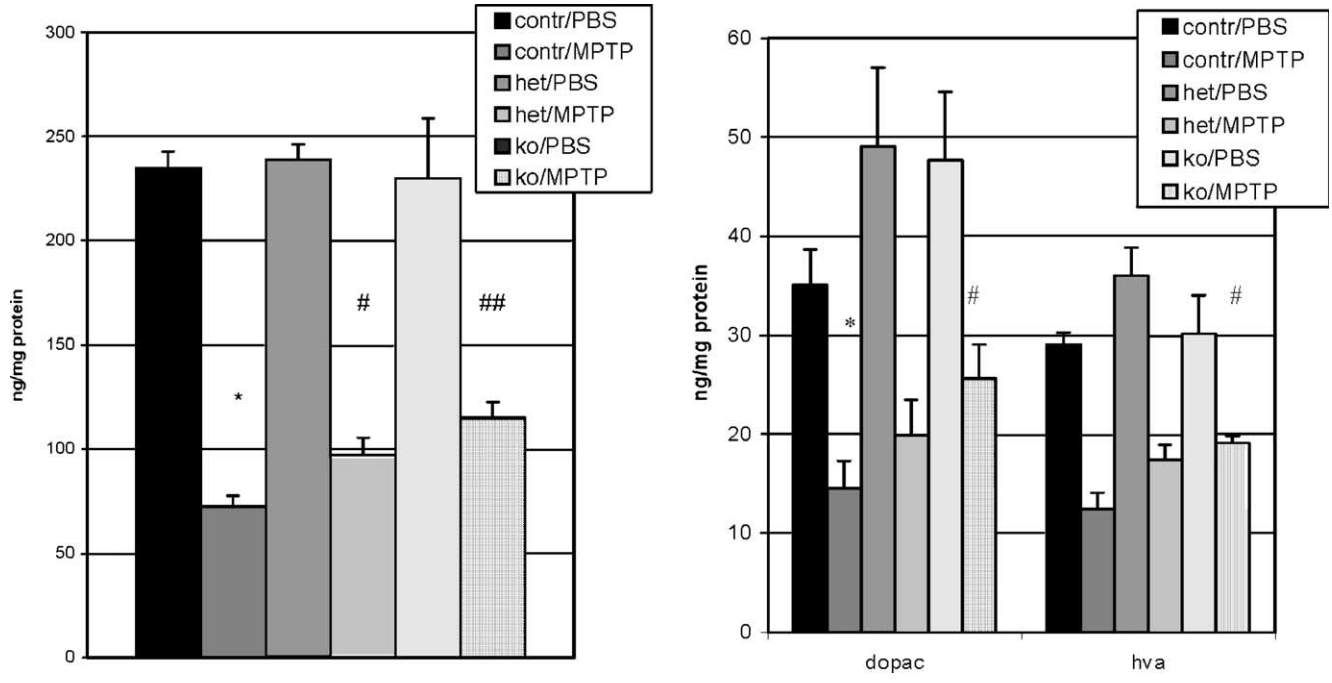

Fig. 1. Effects of MPTP on dopamine, DOPAC, and HVA in wild-type control, α-synuclein heterozygous knockout, and homozygous knockout mice. The lack of α-synuclein protein provided a dose-dependent neuroprotection against MPTP-induced dopamine DOPAC a HVA depletion $^{*}P < 0.001$, as compared to PBS-treated wild-type animals; $^{\#}P < 0.05$ compared to MPTP-treated wild-type animal $^{\#\#}P < 0.002$, as compared to MPTP-treated wild-type mice by ANOVA with Fisher PLSD post hoc test. Het: heterozygous knockout mice, ko: homozygous knockout mice.

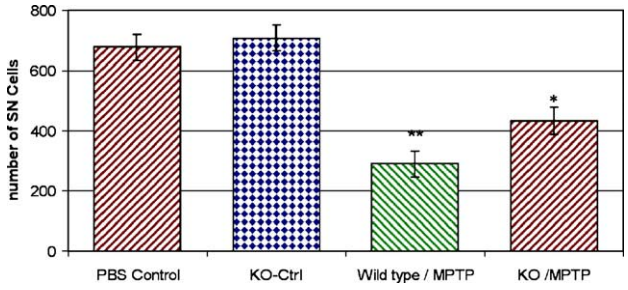

Fig. 2. Effects of MPTP on numbers of substantia nigra DAT immunoreactive neurons. There were no differences in the knockout mice treated with vehicle as compared to wild type, but there was a significant protection against cell loss in knockout mice as compared to wild type following MPTP. $^{**}P < 0.01$ compared to wild type with PBS; $^{*}P < 0.05$ compared to MPTP-treated wild type by ANOVA with Fisher PLSD post hoc test.

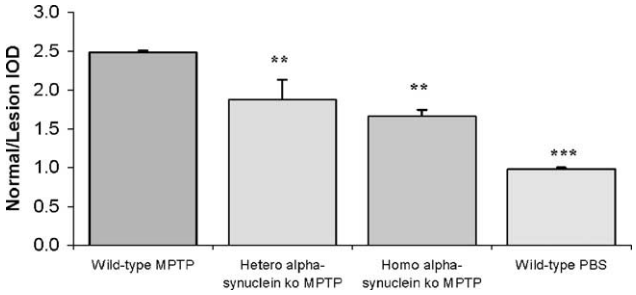

Fig. 4. Cross-sectional area of MPTP induced loss of DAT immunoreactivity in the striatum. The lesion areas were significantly attenuated in both heterozygous and homozygous α-synuclein knockout mice as compared to wild type treated with MPTP. $^{**}P < 0.01$, $^{***}P < 0.001$.

MPTP was not caused by an alteration in uptake or metabolism of MPTP to MPP^+ because striatal MPP^+ levels did not significantly differ at 90 min after MPTP administration (MPP^+ 4.74 ± 1.48 ng/mg protein in controls and 5.12 ± 1.91 ng/mg protein in homozygous knockout mice).

As shown in Fig. 2, the number of DAT immunoreactive neurons in the substantia nigra pars compacta did not differ at baseline in wild-type mice treated with phosphate-buffered saline (PBS) as compared to α-synuclein homozygous knockout mice. Following administration of MPTP, there was a significant depletion of DAT immunoreactive neurons in the wild-type mice, which was significantly attenuated in the homozygous α-synuclein knockout mice (Figs. 2 and 3). Nissl staining confirmed these findings (data not shown). We also examined the area of MPTP-induced loss of DAT immunoreactivity in the striatum. This was significantly attenuated in both the heterozygous and the homozygous α-synuclein knockout mice, consistent with the dopamine measurements (Fig. 4).

We also examined whether a deficiency of α-synuclein would protect against striatal lesions produced by either malonate or 3-NP. As shown in Figs. 5 and 6, striatal lesions produced by malonate were significantly attenuated by 35% in the homozygous α-synucelin knockout mice. The striatal lesion volumes produced by 3-NP are shown in Fig. 7. Administration of 3-NP produced striatal lesions in the wild-type mice that were dose-dependently significantly attenuated in the heterozygous and homozygous α-synuclein knockout mice.

To confirm that there was no alteration in uptake or metabolism of 3-NP in the α-synuclein-deficient mice, we measured its effects on SDH activity in both cerebral cortex and striatum of 3-NP treated mice versus vehicle. In all instances, SDH was reduced approximately 80% from control values. The values in cortex in controls, α-synuclein$^{+/-}$ and α-synuclein$^{-/-}$ mice treated with 3-NP did not significantly differ (0.34 ± 0.04, 0.43 ± 0.04 and 0.30 ± 0.02 nmol DCPIP/min/mg of tissue wet weight. Similarly, there were no significant differences in the striatum (0.25 ± 0.03, 0.27 ± 0.02, and 0.25 ± 0.02 nmol DCPIP/min/mg of tissue wet weight.

Conversion of 4HBA to 3,4DHBA in α-synuclein knockout mice after administration of 3-NP is shown in Fig. 8. The conversion of 4HBA to 3,4DHBA is a well-described marker of oxidative stress mediated by OH· radicals. Under physiological conditions, there was no difference in the 3,4DHBA/4HBA ratio in the striata of wild-type and homozygous knockout mice. Administration of 3-NP resulted in a significant increase in the ratio of 3,4DHBA to 4HBA in wild-type mice, which was dose

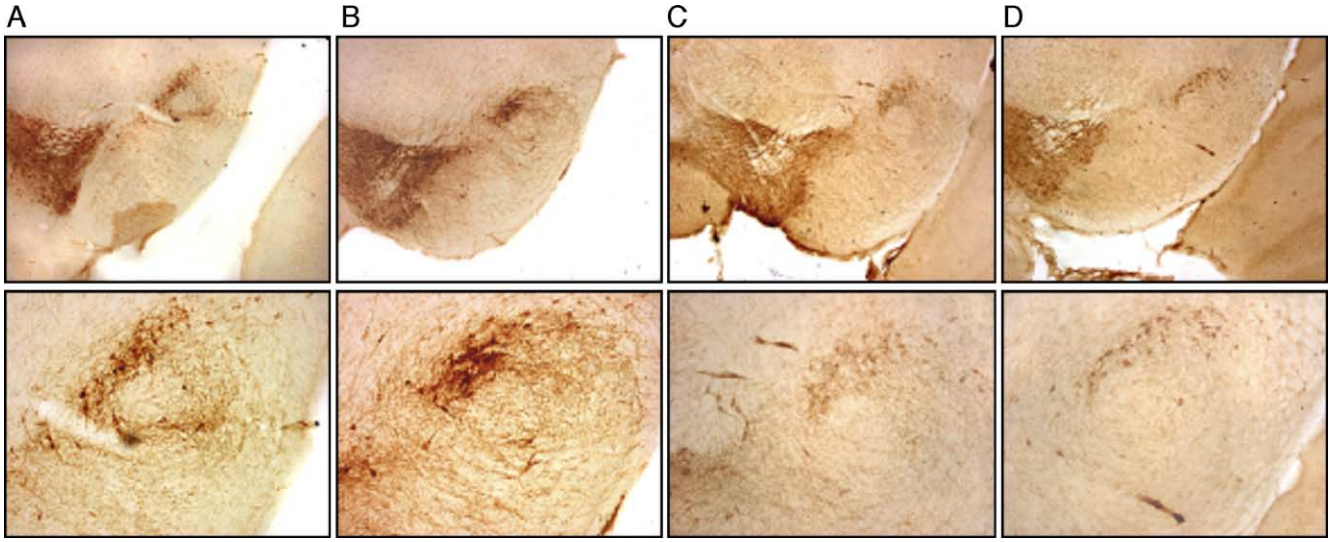

Fig. 3. Representative sections shown at low magnification (top) and high magnification of DAT immunostained neurons in the substantia nigra of wild-type mice treated with PBS (A), α-synuclein$^{-/-}$ mice treated with PBS (B), wild-type mice treated with MPTP (C) and α-synuclein$^{-/-}$ mice treated with MPTP (D).

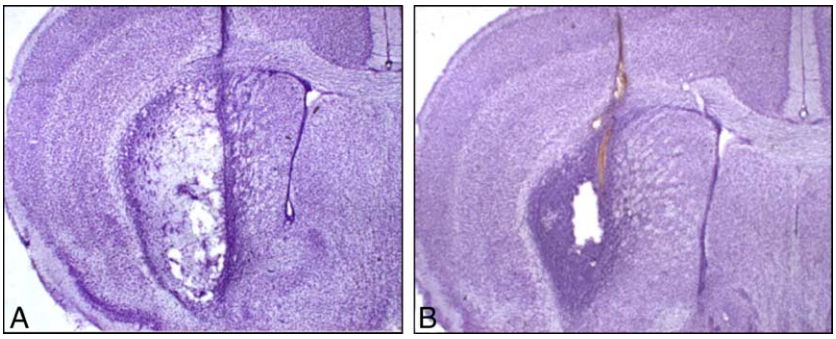

Fig. 5. Nissl stained sections showing a malonate lesion in a wild-type mouse (A) and in α-synuclein$^{-/-}$ mouse (B).

dependently significantly attenuated in heterozygous and homozygous knockout mice ($P < 0.05$ and $P < 0.02$ respectively).

Discussion

In the present experiments, we examined the susceptibility of α-synuclein-deficient mice to the neurotoxin MPTP, which has been used to model PD in mice (Beal, 2001). We found that α-synuclein mice are resistant to both dopamine depletion and loss of DAT immunostained neurons in the substantia nigra pars compacta. This resistance did not appear to be due to altered uptake or processing of MPTP, since we found no significant differences in MPP^+ levels in the mutant as compared to control mice at 90 min. Sequestration of MPP^+ into vesicles is therefore unlikely to account for the protection we observed.

In the present experiments, we also examined the susceptibility of α-synuclein-deficient mice to the mitochondrial toxins malonate and 3-NP. Malonate and 3-NP are respectively reversible and irreversible inhibitors of succinate dehydrogenase, which replicate many of the characteristic pathologic and phenotypic features of HD (Beal et al., 1993; Brouillet and Hantraye, 1995). We found that the striatal lesions produced by both of these toxins were significantly decreased in α-synuclein deficient as compared to control mice. These findings, therefore, show that α-synuclein deficiency protects not only against MPTP but also against direct acting mitochondrial toxins. The resistance to 3-NP was not due to any difference in uptake or metabolism, since the extent of SDH inhibition in both the cerebral cortex and striatum did not significantly differ between control, hemizygous, and homozygous α-synuclein knockout mice. In the case of both MPTP and 3-NP, we found that the protection was gene dosage-dependent since homozygote knockout mice showed greater protection than heterozygote knockout mice.

We also examined whether the α-synuclein-deficient mice produce less reactive oxygen species. We previously showed that 3-NP increases hydroxyl radical generation, and that 3-NP induced striatal lesions are significantly attenuated by free radical scavengers (Schulz et al., 1996). Furthermore, 3-NP lesions are exacerbated in mice deficient in free radical scavenging enzymes (Klivenyi et al., 2000; Andreassen et al., 2001). In the present experiments, we found that α-synuclein-deficient mice show reduced hydroxyl radical generation following intrastriatal administration of 3-NP, consistent with the neuroprotective effects we observed.

How might α-synuclein modulate oxidative damage. α-Synuclein appears to be important in vesicular loading of dopamine (Abeliovich et al., 2000). MPTP induced release of dopamine was reduced in another line of α-synuclein-deficient mice (Dauer et al., 2002). When α-synuclein expression is reduced in cultured rat neurons, the number of vesicles in the distal pool of the presynaptic terminal is reduced (Murphy et al., 2000). In the α-synuclein mice tissue (intracellular), dopamine was reduced (Abeliovich et al., 2000). Thus, there may be reduced cytoplasmic dopamine in the α-synuclein-deficient mice. The importance of cytoplasmic dopamine

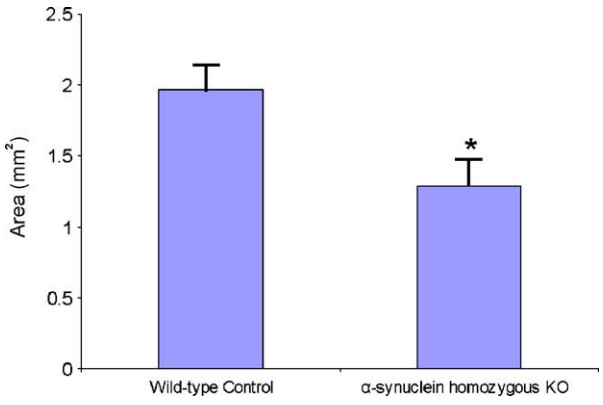

Fig. 6. Striatal lesion volume produced by malonate in wild type compared to homozygous α-synuclein knockout mice. The striatal lesion volumes were significantly attenuated. *$P < 0.05$.

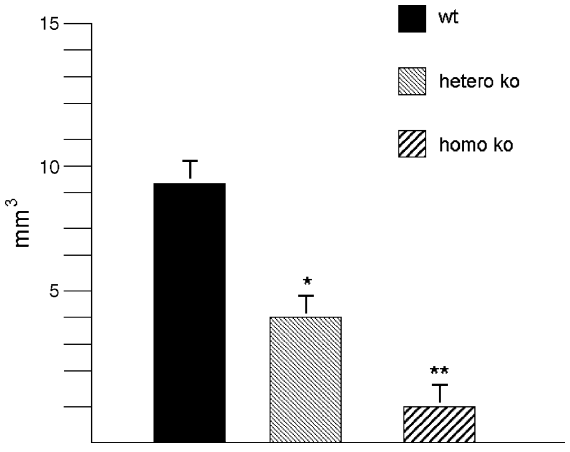

Fig. 7. Striatal lesion volumes in wild-type heterozygous knockout and homozygous knockout mice following administration of 3-NP. The striatal lesion volumes were significantly attenuated in both the heterozygous knockout and the homozygous knockout mice. *$P < 0.05$, **$P < 0.01$.

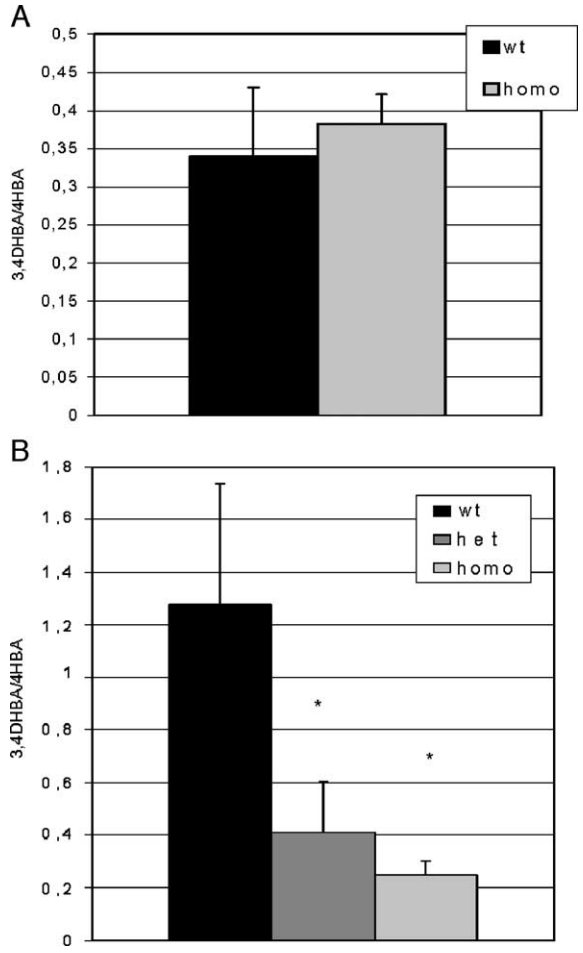

Fig. 8. Conversion of 4HBA to 3,4DHBA in α-synuclein knockout mice under physiological conditions and after systemic administration of 3-NP. There was no difference in the 3,4DHBA/4HBA ratio under physiological conditions. Administration of 3-NP resulted in a significant increase in the ratio of 3,4DHBA to 4HBA in wild-type mice, which was significantly attenuated in heterozygous and homozygous knockout mice. (A) Basal levels; (B) after 3-NP. *$P < 0.05$, compared to wild-type mice. Wt: wild type, het: heterozygous knockout mice, homo: homozygous knockout mice.

to PD cell death is supported by the finding that the dopaminergic neurons of the ventral tegmental area, which are resistant as compared to the substantia nigra, express high levels of the vesicular monoamine transporter, which promotes vesicular sequestration of dopamine, and low levels of the dopamine transporter which pumps dopamine into the cytoplasm (Takahashi et al., 1997). Cytoplasmic dopamine may autooxidize, contributing to the production of reactive oxygen species (Sulzer et al., 2000). Accumulation of α-synuclein in cultured human dopaminergic neurons results in apoptosis which is dependent on dopamine and the production of reactive oxygen species (Xu et al., 2002).

A reduction in release of dopamine may explain the neuroprotection seen in the α-synuclein mice against both malonate and 3-NP neurotoxicity. Striatal lesions produced by malonate and 3-NP, as well as the generation of reactive oxygen species, are significantly attenuated in rats with 6-hdyroxydopamine lesions of the striatum or pharmacologic depletion of dopamine (Maragos et al., 1998; Reynolds et al., 1998; Xia et al., 2001). Furthermore, systemic or intrastriatal administration of L-DOPA or dopamine, respectively, restores malonate toxicity and generation of reactive oxygen species in 6-hydroxydopamine lesioned rats (Xia et al., 2001), and dopamine transporter knockout mice are hypersensitive to 3-NP-induced striatal damage (Fernagut et al., 2002).

Several prior studies showed that α-synuclein-deficient mice were resistant to MPTP neurotoxicity. The initial study suggested that a lack of α-synuclein interfered with the ability of MPP^+ to block complex I, perhaps by increasing monoamine vesicular transport (Dauer et al., 2002). In another study of chronic MPTP administration, mice lacking α-synuclein had an attenuated loss of dopamine but no loss of striatal vesicular monoamine transporter protein (VMAT-2) (Drolet et al., 2004). Despite the attenuated toxicity, elevated lactate concentrations were observed in the α-synuclein knockout mice, arguing against impaired ability of MPP^+ to impair mitochondrial energy production. Continuous MPTP infusion by Alzet pump produced long-lasting activation of glucose uptake, and impairment of the ubiquitin–proteosome system (Fornai et al., 2004). In mice lacking α-synuclein MPTP induced metabolic activation, but behavioral symptoms and loss of dopamine neurons were almost completely abrogated, as was the production of α-synuclein inclusion bodies (Fornai et al., 2004). Our present findings that mice lacking α-synuclein are resistant not only to MPTP but also to malonate and 3-NP, and that they exhibit reduced generation of reactive oxygen species, suggest that α-synuclein deficiency results in neuroprotection by reducing oxidative stress. This is consistent with observations that overexpression of α-synuclein increases oxidative stress (Orth et al., 2004). Increased α-synuclein can inhibit the proteosome which then leads to oxidative damage in vitro (Sullivan et al., 2004).

The present findings are consistent with a role of α-synuclein in modulating dopamine release and oxidative damage in PD. There may be a complex interaction as suggested by the observation that oxidative forms of dopamine can promote α-synuclein protofibril generation (Conway et al., 2001). Expression of mutant α-synuclein causes increased susceptibility to dopamine toxicity, and an α-synuclein fragment produces neurotoxicity to dopaminergic neurons both in vitro and in vivo (Forloni et al., 2000; Tabrizi et al., 2000; Xu et al., 2002). Furthermore, administration of rotenone, a selective mitochondrial complex I inhibitor which generates ROS, can produce, selective damage to substantia nigra neurons, and α-synuclein positive Lewy bodies (Betarbet et al., 2000). MPTP upregulates α-synuclein expression in both mice and primates (Kowall et al., 2000; Vila et al., 2000; Fornai et al., 2004). Paraquat which produces oxidative damage and is implicated PD pathogenesis also upregulates α-synuclein (Manning-Bog et al., 2002). Lastly, oxidative damage may contribute to Lewy body generation (Giasson et al., 2000). The mutations in α-synuclein in familial PD may promote the ability of α-synuclein to generate protofibrils. In sporadic PD, exposure to environmental toxins may produce oxidative damage, promote α-synuclein expression and aggregation, which is then exacerbated by dopamine, and directly contribute on degeneration of substantia nigra neurons.

Acknowledgments

The secretarial assistance of Sharon Melanson and Greta Strong is gratefully acknowledged. This work was supported by grants from the Department of Defense, NINDS and the Parkinson's Disease Foundation.

References

Abeliovich, A., Schmitz, Y., Farinas, I., Choi-Lundberg, D., Ho, W.H., Castillo, P.E., Shinsky, N., Verdugo, J.M., Armanini, M., Ryan, A., Hynes, M., Phillips, H., Sulzer, D., Rosenthal, A., 2000. Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. Neuron 25, 239–252.

Andreassen, O.A., Dedeoglu, A., Ferrante, R.J., Jenkins, B.G., Ferrante, K.L., Thomas, M., Friedlich, A., Browne, S.E., Schilling, G., Borchelt, D.R., Hersch, S.M., Ross, C.A., Beal, M.F., 2001. Creatine increase survival and delays motor symptoms in a transgenic animal model of Huntington's disease. Neurobiol. Dis. 8, 479–491.

Arrigon, O., Singer, T., 1962. Limitations of the phenazine methosulfate assay for succinic and related dehydrogenases. Nature 193, 1256–1258.

Beal, M.F., 2001. Experimental models of Parkinson's disease. Nat. Rev., Neurosci. 2, 325–334.

Beal, M.F., Brouillet, E., Jenkins, B., Henshaw, R., Rosen, B., Hyman, B.T., 1993. Age-dependent striatal excitotoxic lesions produced by the endogenous mitochondrial inhibitor malonate. J. Neurochem. 61, 1147–1150.

Betarbet, R., Porter, R.H., Greenamyre, J.T., 2000. GluR1 glutamate receptor subunit is regulated differentially in the primate basal ganglia following nigrostriatal dopamine denervation. J. Neurochem. 74, 1166–1174.

Brouillet, E., Hantraye, P., 1995. Effects of chronic MPTP and 3-nitropropionic acid in nonhuman primates. Curr. Opin. Neurol. 8, 469–473.

Chen, L., Feany, M.B., 2005. alpha-Synuclein phosphorylation controls neurotoxicity and inclusion formation in a *Drosophila* model of Parkinson's disease. Nat. Neurosci. 8, 657–663.

Clayton, D.F., George, J.M., 1999. Synucleins in synaptic plasticity and neurodegenerative disorders. J. Neurosci. Res. 58, 120–129.

Cole, N.B., Murphy, D.D., 2002. The cell biology of a-synuclein. Neuro-Molec. Med. 1, 95–109.

Conway, K.A., Lee, S.J., Rochet, J.C., Ding, T.T., Williamson, R.E., Lansbury Jr., P.T., 2000. Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy. Proc. Natl. Acad. Sci. U. S. A. 97, 571–576.

Conway, K.A., Rochet, J.C., Bieganski, R.M., Lansbury Jr., P.T., 2001. Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct. Science 294, 1346–1349.

Dauer, W., Kholodilov, N., Vila, M., Trillat, A.C., Goodchild, R., Larsen, K.E., Staal, R., Tieu, K., Schmitz, Y., Yuan, C.A., Rocha, M., Jackson-Lewis, V., Hersch, S., Sulzer, D., Przedborski, S., Burke, R., Hen, R., 2002. Resistance of a-synuclein null mice to the parkinsonsian neurotoxin MPTP. Proc. Natl. Acad. Sci. U. S. A. 99, 14524–14529.

Drolet, R.E., Behrouz, B., Lookingland, K.J., Goudreau, J.L., 2004. Mice lacking a-synuclein have an attenuated loss of striatal dopamine following prolonged chronic MPTP administration. NeuroToxicology 25, 761–769.

Feany, M.B., Bender, W.W., 2000. A *Drosophila* model of Parkinson's disease. Nature 404, 298–394.

Fernagut, P.O., Diguet, E., Jaber, M., Bioulac, B., Tison, F., 2002. Dopamine transporter knock-out mice are hypersensitive to 3-nitropropionic acid-induced striatal damage. Eur. J. Neurosci. 15, 2053–2056.

Forloni, G., Bertani, I., Calella, A.M., Thaler, F., Invernizzi, R., 2000. Alpha-synuclein and Parkinson's disease: selective neurodegenerative effect of alpha-synuclein fragment on dopaminergic neurons in vitro and in vivo. Ann. Neurol. 47, 632–640.

Fornai, F., Schulter, O.M., Lenzi, P., Gesi, M., Ruffoli, R., Ferrucci, M., Lazzeri, G., Busceti, C.L., Pontarelli, F., Battaglia, G., Pellegrini, A., Nicoletti, F., Ruggieri, S., Paparelli, A., Sudhof, T.C., 2004. Parkinson-like syndrome induced by continuous MPTP infusion: convergent roles of the ubiqutin–proteasome system and a-synuclein. Proc. Natl. Acad. Sci. 102, 3413–3418.

Furlong, R.A., Narain, Y., Rankin, J., Wyttenbach, A., Rubinsztein, D.C., 2000. alpha-synuclein overexpression promotes aggregation of mutant huntingtin. Biochem. J. 346, 577–581.

Giasson, B.I., Duda, J.E., Murray, I.V., Chen, Q., Souza, J.M., Hurtig, H.I., Ischiropoulos, H., Trojanowski, J.Q., Lee, V.M., 2000. Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. Science 290, 985–989.

Giasson, B.I., Duda, J.E., Quinn, S.M., Zhang, B., Trojanowski, J.Q., Lee, V.M., 2002. Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. Neuron 34, 521–533.

Goedert, M., 2001. Alpha-synuclein and neurodegenerative diseases. Nat. Rev., Neurosci. 2, 492–501.

Hsu, L.J., Sagara, Y., Arroyo, A., Rockenstein, E., Sisk, A., Mallory, M., Wong, J., Takenouchi, T., Hashimoto, M., Masliah, E., 2000. α-Synuclein promotes mitochondrial deficit and oxidative stress. Am. J. Pathol. 157, 401–410.

Kahle, P.J., Neumann, M., Ozmen, L., Muller, V., Jacobsen, H., Schindzielorz, A., Okochi, M., Leimer, U., van Der Putten, H., Probst, A., Kremmer, E., Kretzschmar, H.A., Haass, C., 2000. Subcellular localization of wild-type and Parkinson's disease-associated mutant alpha-synuclein in human and transgenic mouse brain. J. Neurosci. 20, 6365–6373.

Kirik, D., Georgievska, B., Burger, C., Winkler, C., Muzyczka, N., Mandel, R.J., Bjorklund, A., 2002. Reversal of motor impairments in parkinsonian rats by continuous intrastriatal delivery of L-DOPA using rAAV-mediated gene transfer. Proc. Natl. Acad. Sci. U. S. A. 99, 4708–4713.

Klivenyi, P., Andreassen, O.A., Ferrante, R.J., Dedeoglu, A., Mueller, G., Lancelot, E., Bogdanov, M., Andersen, J.K., Jiang, D., Beal, M.F., 2000. Mice deficient in cellular glutathione peroxidase show increased vulnerability to malonate, 3-nitropropionic acid, and 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. J. Neurosci. 20, 1–7.

Ko, L., Mehta, N.D., Farrer, M., Easson, C., Hussey, J., Yen, S., Hardy, J., Yen, S.H., 2000. Sensitization of neuronal cells to oxidative stress with mutated human alpha-synuclein. J. Neurochem. 75, 2546–2554.

Kowall, N.W., Hantraye, P., Brouillet, E., Beal, M.F., McKee, A.C., Ferrante, R.J., 2000. MPTP induces alpha-synuclein aggregation in the substantia nigra of baboons. NeuroReport 11, 211–213.

Kruger, R., Vieira-Saecker, A.M., Kuhn, W., Berg, D., Muller, T., Kuhnl, N., Fuchs, G.A., Storch, A., Hungs, M., Woitalla, D., Przuntek, H., Epplen, J.T., Schols, L., Riess, O., 1999. Increased susceptibility to sporadic Parkinson's disease by a certain combined alpha-synuclein/a-polipoprotein E genotype. Ann. Neurol. 45, 611–617.

Lee, F.J., Liu, F., Pristupa, Z.B., Niznik, H.B., 2001a. Direct binding and functional coupling of alpha-synuclein to the dopamine transporters accelerate dopamine-induced apoptosis. FASEB J. 15, 916–926.

Lee, M., Hyun, D.H., Halliwell, B., Jenner, P., 2001b. Effect of the overexpression of wild-type or mutant a-synuclein on cell susceptibility to insult. J. Neurochem. 76, 998–1009.

Manning-Bog, A.B., McCormack, A.L., Li, J., Uversky, V.N., Fink, A.L., Di Monte, D.A., 2002. The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: paraquat and alpha-synuclein. J. Biol. Chem. 377, 1641–1644.

Maragos, W.F., Jakel, R.J., Pang, Z., Geddes, J.W., 1998. 6-Hydroxydopamine injections into the nigrostriatal pathway attenuate striatal malonate and 3-nitropropionic acid lesions. Exp. Neurol. 154, 637–644.

Mezey, E., Toth, Z.E., Cortright, D.N., Arzubi, M.K., Krause, J.E., Elde, R., Guo, A., Blumberg, P.M., Szallasi, A., 2000. Distribution of mRNA for vanilloid receptor subtype 1 (VR1), and VR1-like immunoreactivity, in the central nervous system of the rat and human. Proc. Natl. Acad. Sci. U. S. A. 97, 3655–3660.

Murphy, D.D., Rueter, S.M., Trojanowski, J.Q., Lee, V.M., 2000. Synucleins are developmentally expressed, and alpha-synuclein regulates the size of the presynaptic vesicular pool in primary hippocampal neurons. J. Neurosci. 20, 3214–3220.

Orth, M., Tabrizi, S.J., Tomlinson, C., Messmer, K., Korlipara, L.V.P., Schapira, A.H.V., Cooper, J.M., 2004. G209A mutant alpha synuclein

expression specifically enhances dopamine induced oxidative damage. Neurochem. Int. 45, 669–676.
Ostrerova-Golts, N., Petrucelli, L., Hardy, J., Lee, J.M., Farer, M., Wolozin, B., 2000. The A53T alpha-synuclein mutation increases iron-dependent aggregation and toxicity. J. Neurosci. 20, 6048–6054.
Paxinou, E., Chen, Q., Weisse, M., Giasson, B.I., Norris, E.H., Rueter, S.M., Trojanowski, J.Q., Lee, V.M., Ischiropoulos, H., 2001. Induction of a-synuclein aggregation by intracellular nitrative insult. J. Neurosci. 21, 8053–8061.
Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E.S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W.G., Lazzarini, A.M., Duvoisin, R.C., Di Iorio, G., Golbe, L.I., Nussbaum, R.L., 1997. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 276, 2045–2047.
Reynolds, D.S., Carter, R.J., Morton, A.J., 1998. Dopamine modulates the susceptibility of striatal neurons to 3-nitropropionic acid in the rat model of Huntington's disease. J. Neurosci. 18, 10116–10127.
Richfield, E.K., Thiruchelvam, M.J., Cory-Slechta, D.A., Wuertzer, C., Gainetdinov, R.R., Caron, M.G., DiMonte, D.A., Federoff, H.J., 2002. Behavioral and neurochemical effects of wild-type and mutated human alpha-synuclein in transgenic mice. Exp. Neurol. 175, 35–48.
Schulz, J.B., Henshaw, D.R., MacGarvey, U., Beal, M.F., 1996. Involvement of oxidative stress in 3-nitropropionic acid neurotoxicity. Neurochem. Int. 29, 167–171.
Souza, J.M., Giasson, B.I., Chen, Q., Lee, V.M., Ischiropoulos, H., 2000. Dityrosine cross-linking promotes formation of stable alpha-synuclein polymers. Implication of nitrative and oxidative stress in the pathogenesis of neurodegenerative synucleinopathies. J. Biol. Chem. 275, 18344–18349.
Spillantini, M.G., Crowther, R.A., Jakes, R., Hasegawa, M., Goedert, M., 1998. Alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. Proc. Natl. Acad. Sci. U. S. A. 95, 6469–6473.
Sullivan, P.G., Dragicevic, N.B., Deng, J.H., Bai, Y., Dimayuga, E., Ding, Q., Chen, Q., Bruce-Keller, A.J., Keller, J.N., 2004. Proteasome inhibition alters neural mitochondrial homeostasis and mitochondria turnover. J. Biol. Chem. 279, 20699–20707.
Sulzer, D., Bogulavsky, J., Larsen, K.E., Behr, G., Karatekin, E., Kleinman, M.H., Turro, N., Krantz, D., Edwards, R.H., Greene, L.A., Zecca, L., 2000. Neuromelanin biosynthesis is driven by excess cytosolic catecholamines not accumulated by synaptic vesicles. Proc. Natl. Acad. Sci. U. S. A. 97, 11869–11874.
Tabrizi, S.J., Orth, M., Wilkinson, J.M., Taanman, J.W., Warner, T.T., Cooper, J.M., Schapira, A.H., 2000. Expression of mutant alpha-synuclein causes increased susceptibility to dopamine toxicity. Hum. Mol. Genet. 9, 2683–2689.
Takahashi, N., Miner, L.L., Sora, I., Ujike, H., Revay, R.S., Kostic, V., Jackson-Lewis, V., Przedborski, S., Uhl, G.R., 1997. VMAT2 knockout mice: heterozygotes display reduced amphetamine-conditioned reward, enhanced amphetamine locomotion, and enhanced MPTP toxicity. Proc. Natl. Acad. Sci. U. S. A. 94, 9938–9943.
Tu, P.H., Galvin, J.E., Baba, M., Giasson, B., Tomita, T., Leight, S., Nakajo, S., Iwatsubo, T., Trojanowski, J.Q., Lee, V.M., 1998. Glial cytoplasmic inclusions in white matter oligodendrocytes of multiple system atrophy brains contain insoluble alpha-synuclein. Ann. Neurol. 44, 415–422.
Vila, M., Vukosavic, S., Jackson-Lewis, V., Neystat, M., Jakowec, M., Przedborski, S., 2000. Alpha-synuclein up-regulation in substantia nigra dopaminergic neurons following administration of the parkinsonian toxin MPTP. J. Neurochem. 74, 721–729.
Xia, X.G., Schmidt, N., Teismann, P., Ferger, B., Schulz, J.B., 2001. Dopamine mediates striatal malonate toxicity via dopamine transporter-dependent generation of reactive oxygen species and D2 but not D1 receptor activation. J. Neurochem. 79, 63–70.
Xu, J., Kao, S.Y., Lee, F.J., Song, W., Jin, L.W., Yanker, B.A., 2002. Dopamine-dependent neurotoxicity of alpha-synuclein: a mechanism for selective neurodegeneration in Parkinson's disease. Nat. Med. 8, 600–606.