

RESEARCH PAPER

The regulation of catalase activity by PPAR γ is affected by α -synuclein

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Introduction

Parkinson's disease (PD) is principally a movement disorder involving progressive degeneration of dopaminergic neurons in the substantia nigra. In addition to motor symptoms, PD patients may also suffer from nonmotor symptoms, attributed to degeneration of non-dopaminergic neuronal populations.

There is ample evidence of oxidative injury in brains affected with PD. Specifically, the levels of several oxidative stress markers are increased in the substantia nigra of post mortem PD brains.^{1–3} Furthermore, the activity of catalase and glutathione peroxidase, two enzymes responsible for the elimination of reactive oxygen species (ROS), is reduced in PD brains.^{4,5} Importantly, it is currently

Abstract

Objective: While evidence for oxidative injury is frequently detected in brains of humans affected by Parkinson's disease (PD) and in relevant animal models, there is uncertainty regarding its cause. We tested the potential role of catalase in the oxidative injury that characterizes PD. **Methods:** Utilizing brains of A53T α -Syn and ntg mice, and cultured cells, we analyzed catalase activity and expression, and performed biochemical analyses of peroxisomal metabolites. **Results:** Lower catalase expression and lower activity levels were detected in A53T α -Syn brains and α -Syn-expressing cells. The effect on catalase activity was independent of disease progression, represented by mouse age and α -Syn mutation, suggesting a potential physiological function for α -Syn. Notably, catalase activity and expression were unaffected in brains of mice modeling Alzheimer's disease. Moreover, we found that α -Syn expression downregulate the peroxisome proliferator-activated receptor (PPAR) γ , which controls catalase transcription. Importantly, activation of either PPAR γ 2, PPAR α or retinoic X receptor eliminated the inhibiting effect of α -Syn on catalase activity. In addition, activation of these nuclear receptors enhanced the accumulation of soluble α -Syn oligomers, resulting in a positive association between the degree of soluble α -Syn oligomers and catalase activity. Of note, a comprehensive biochemical analysis of specific peroxisomal metabolites indicated no signs of dysfunction in specific peroxisomal activities in brains of A53T α -Syn mice. **Interpretation:** Our results suggest that α -Syn expression may interfere with the complex and overlapping network of nuclear receptors transcription activation. In result, catalase activity is affected through mechanisms involved in the regulation of soluble α -Syn oligomers.

unknown whether oxidative damage in PD results from increased production or decreased clearance of oxidants (reviewed by⁶).

α -Synuclein (α -Syn) protein is critically implicated in the genetics and cytopathology of PD reviewed by^{7,8}. α -Syn's cytotoxicity is associated with its oligomerization, aggregation and finally, its deposition in Lewy bodies and Lewy neurites, the histopathologic hallmarks of PD and related synucleinopathies.^{9–11} Nevertheless, it is not clear whether this is the cause or the consequence of an earlier neurotoxic event. Numerous factors enhance α -Syn cytotoxicity, including oxidative stress and brain lipid modifications.^{6,12}

In a recent report, we have shown a role for retinoic X receptor (RXR) and peroxisome proliferator-activated

receptor $\gamma 2$ (PPAR $\gamma 2$) in α -Syn oligomerization and suggested that the polyunsaturated fatty acid, docosahexaenoic acid (22:6), enhanced α -Syn oligomerization by acting as an activating ligand for these members of the nuclear receptor (NR) family of transcription factors.¹³

Catalase transcription is regulated by PPAR γ . A putative functional PPAR response element (PPRE) was identified at the promoter region of rat catalase gene.¹⁴ Activation of PPAR γ by a specific agonist further enhances catalase activity and protects neurons from oxidative stress.¹⁵

We now report lower catalase expression and lower activity levels in brains of A53T α -Syn transgenic mice and in MN9D dopaminergic cells overexpressing α -Syn. In addition, we found evidence for down regulation of PPAR γ by α -Syn. Activation of PPAR $\gamma 2$, PPAR α or RXR eliminated the inhibiting effect of α -Syn. Importantly, the activation of these NRs enhanced the occurrence of soluble α -Syn oligomers. On the basis of these results, we now suggest that α -Syn downregulates catalase expression and activity, by influencing the fine balance of the overlapping network of NR activation, which also controls its conformation.

Materials and Methods

Mice

The A53T α -Syn transgenic¹⁶ and APP/PS1¹⁷ mouse lines were purchased from Jackson Laboratories (Bar Harbor, ME). The A53T α -Syn mice were bred to homozygosity. Similar to the original description, the colony we maintained remained healthy up to the age of ~7–8 months. At 9–10 months of age, a portion of the mice in the colony began to develop motor and behavioral abnormalities. The percent of affected animals grew with age. Motor and behavior abnormalities were similar to the original description of these mice.¹³

In parallel, we maintained control mice with a similar genetic background, (C57Bl/6/C3H; Jackson Laboratories, Bar Harbor, ME). The APP/PS1^{+/-} mice were compared to their nontransgenic (ntg) littermates. 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 Authority of Biological and Biomedical Models of the Hebrew University of Jerusalem, NIH approval # OPRR-A01-5011 (Permit Number: MD-09-12084).

Cells

The mesencephalic neuronal cell line, MN9D¹⁸ or HeLa cells were transfected with human wild type or A53T α -Syn cDNA in the pCDNA 3.1 vector. Since α -Syn expres-

sion and oligomerization are dynamic in these clones,¹⁹ comparisons were made between clones of wild type and A53T that were maintained in parallel from DNA transfection and selection of stable clones to the actual measurements.¹⁹

Agonist treatments were performed in HeLa cells conditioned with troglitazone or 9-cis retinoic acid in dimethyl sulfoxide (DMSO) for 16 h. Cells were then collected and processed for catalase activity measurements.

Transient expression in HeLa cells was performed using ICAfectin (Tal Ron Biotech, Rehovot, Israel). Transfection of 1×10^5 cells (6 cm dish) was performed with the following cDNAs: human RXR α (obtained from Bruce M. Spiegelman at Harvard Medical School, Boston, MA); human PPAR γ 1 or PPAR γ 2 cDNAs (obtained from Ron Evans at Howard Hughes Medical Institute, Chevy Chase, MD); or mock transfected. Forty-eight hours post DNA transfection, cells were collected and processed for catalase activity.

Peroxisome-enriched fractions

Peroxisome-enriched fractions were obtained as described previously by Kovacs *et al.*,²⁰ with slight modifications. Briefly, the tissue was homogenized in three volumes (w/v) of 50 mmol/L 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.4, 250 mmol/L sucrose, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1% ethanol (v/v), and protease inhibitors cocktail (Sigma, Rehovot, Israel) by five strokes of Teflon homogenizer. The homogenate was centrifuged at 1000g for 10 min at 4°C. The supernatant was removed and kept in a clean tube; the pellet was resuspended in the above buffer and centrifuged at 600g for 10 min at 4°C. This procedure was repeated once again. The final pellet consisting of nuclei, large myelin fragments, and tissue debris was discarded. The combined supernatants were made up to a volume of 10% (w/v) and centrifuged at 5500g for 10 min to remove mitochondria. The resulting supernatant was centrifuged at 18,000g for 30 min to yield an enriched peroxisomal fraction (pellet). Frozen aliquots were immediately stored at -70°C until use. Protein content was determined by the Bradford method.²¹

Immunohistochemistry

Immunohistochemistry was performed as previously described.^{13,22} Briefly, mice were anesthetized with an intraperitoneal overdose injection of sodium pentobarbital (1 mL/1.5 kg) and perfused with phosphate-buffered saline buffered formalin (4%). Following surgical removal, the brains were fixed for another 24 h in the formalin solution. Brain sections (5 μ m) were deparaffinized in

xylene followed by rehydration in descending ethanol concentrations. Endogenous peroxidase activity was reduced by incubation in methanol/H₂O₂ (150 mL methanol and 30 mL of 30% H₂O₂). Antigen retrieval was obtained with 0.01% trypsin digestion for 10 min at 37°C, followed by microwaving in 10 mmol/L citrate buffer at pH 6.0 for 15 min at 800W in a conventional household microwave oven.²³ Nonspecific binding sites were blocked with casein solution (CAS-Block; Invitrogen, Jerusalem, Israel) for 30 min and sections were probed overnight with primary antibodies: rabbit anti-catalase (1:200; Rockland Immunochemicals, Gilbertsville, PA); rabbit anti-Pex14, 1:300 (a gift from D. Crane, Griffith University, Australia); mouse anti-NeuN (1:700; Millipore, Billerica, MA). Slides were then washed and reacted with a secondary antibody, anti-mouse Cy2 (1:300; Jackson Laboratories, ME) and anti-rabbit Cy5 (1:300; Jackson Laboratories, ME). The stained sections were observed by confocal microscopy. Quantifications were blinded for genotypes. Comparisons between genotypes (age-matched) were made only between slides that were stained and handled in parallel. Image series analyzed with Image pro plus 6.3 program (Media Cybernetics, Bethesda, MD). The detection of endogenous and transgenic α -Syn was performed as described before.¹³

Confocal microscopy

Images were captured using a Zeiss LSM 710 Axio Observer. Z1 laser scanning (Getter BioMed, Petah Tikva, Israel). The system is equipped with an argon laser 488 excitation with 494–630 nm pass barrier filter, diode 405–30 laser excitation with 410–483 nm pass barrier filter and HeNe 633 lasers, excitation with 638–759 nm pass barrier filter. Fluorescence was collected simultaneously with differential interference contrast (DIC) images using a transmitted light detector. The fluorescence signal was collected by employing a “Plan-Apochromat” 63x/1.40 Oil DIC M27 or “Plan-Neofluar” 40x/1.30 Oil DIC M27 (Zeiss, Oberkochen, Germany). In each experiment, exciting laser, intensity, background levels, photo multiplier tube (PMT) gain, contrast and electronic zoom size were maintained at the same level. For each antibody, the background was subtracted (determined by a negative control consisting of a secondary antibody alone). The zoom of each picture was obtained by choosing the plane with the greatest fluorescent signal.

Catalase enzymatic assay

Catalase activity was measured using Amplex Red Catalase Assay kit (Molecular Probes, Invitrogen, Israel). One brain hemisphere or a 10 cm subconfluent culture dish of

MN9D or HeLa cells (naïve or α -Syn overexpressing) was homogenized 1:10 (w/v) in a buffer consisting of 50 mmol/L MOPS, pH 7.4, 250 mmol/L sucrose, 1 mmol/L EDTA, 0.1% ethanol (v/v), and a protease inhibitors cocktail (Sigma, Rehovot, Israel). Homogenization was performed by a Dounce homogenizer, followed by seven passages through a 27 g needle. The homogenate was centrifuged at 180,000g for 1 h and the supernatants were stored at –80°C in aliquots. Samples were incubated with 40 μ mol/L H₂O₂ for 40 min with or without 3-amino-1,2,4-triazole (3-AT) (20 mmol/L). The non degraded H₂O₂ was measured to determine catalase activity against a standard curve. In the presence of horseradish peroxidase, Amplex Red reacts stoichiometrically with H₂O₂ to generate resorufin, which can be measured at 571 nm (absorption) or 585 nm (fluorescent). Note that the assay determines the elimination of free H₂O₂ from the reaction mix, by binding to catalase, rather than the actual degradation to H₂O and O₂. The assay consists of the ability of catalase to bind hydrogen peroxide (H₂O₂) at high affinity.²⁴

Western blotting

PPAR γ detection: one brain hemisphere from either a ntg or A53T α -Syn tg mouse was homogenized in radio immunoprecipitation assay buffer (50 mmol/L Tris; 150 mmol/L NaCl; 1 mmol/L EDTA; 1% Nonidet P-40; 0.25% Na-deoxycholate; 2 μ g/mL aprotinin; 2 μ g/mL leupeptin; 2 μ g/mL pepstatin A; 0.5 mmol/L phenylmethylsulfonyl fluoride; and 1 mmol/L dithiothreitol). Protein concentration was determined by BCA protein assay (Pierce, Rockford, IL) and protein samples were separated by sodiumdodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) and transferred to a polyvinylidene fluoride membrane. Membranes were blocked in 5% nonfat milk in tris-buffered saline and tween 20. Thereafter, the membranes were immunoblotted with anti-PPAR- γ (1:100) (Santa Cruz, Biochemistry, Santa Cruz, CA; sc-#7273) and anti- β -actin (1:5000) (Sigma #A3853).

Peroxisomal protein detection: samples of 15 μ g protein of peroxisome-enriched fractions (see above) were boiled with Laemmli buffer for 10 min at 100°C prior to separation by SDS-PAGE. The following antibodies were used: rabbit anti-catalase (1:20,000; Rockland), rabbit anti-Pex14 (1:20,000; obtained from Denis Crane, Griffith University), rabbit anti-PMP70 (1:600; ABCam, Tel-Aviv, Israel).

Bands were further visualized using an appropriate horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) and EZ-ECL detection kit (Biological Industries, Bet Haemek, Israel). Quantification of the bands was performed by ImageJ or UN-SCAN-IT gel 3.1 software.

RT-PCR

RNA was isolated using TRI-reagent (Sigma, Rehovot, Israel) and treated with DNase (RQ1 RNase-free DNase; Promega, Bet-Haemek, Israel). cDNA was generated with High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, CA). Quantitative amplification was performed with Power SYBR Green polymerase chain reaction (PCR) Master Mix (Applied Biosystems, Grand Island, NY) and normalized to the levels detected for 18S in the same sample. The following primers were used: mouse ACOX1: TGGGAAGTGCAGCTCAGAGT and CTCTGGCTCGTCTCTTGA; mouse ECO: CAAGTCACAAGTGCCAGAA and CACCAACTCCCTCCAGAAAG; mouse PMP70: AGCTGGGTACATCCTTGAG and CCATCGCCATTCTTTGTTTC; mouse catalase: TCAGGTGCGGACATTCTACA and GAAAAGCTGAGCGTCCTTCA; mouse PPAR γ : GATGTCTCACAATGCCATCAG and TCAGCAGACTCTGGGTTTCAG; mouse 18S: GAGCGAGTGATCACCATCAT and GCCAGAACCTGGCTGTACTT; Human catalase: GGGAGAAGGCAAATCTGTGA and CAG TGA TGAGCGGGTTACAC; human PPAR α : GCA GGA GAT CTA CAG GGA CAT and TTGTAG TGCTGT CAGCTTCAGA; human PPAR β/δ : GAT GGG AAC CAC CCT GTA GA and CTGCTCCATGGCTGATCTC; human PPAR γ : TGATCAAGAAGACGGAGACAGA; and GCAGTGGCTCAGGACTCTCT; human RXR α : GCC GGGCATGAGTTAGTC and GTTCACCTGGGTGGAGAAAT; human G6PD CACCATCTGGTGGCTGTTC and TCACTCTGTTTGGCGGATGTC.

FACS

PPAR γ transcription activity was determined by a green fluorescent protein (GFP) reporter plasmid assays pGreenFire1-PPRE, (TR101PA-1; System Biosciences, Mountain View, CA). Mock-transfected and α -Syn-overexpressing HeLa clones were transfected with the reporter plasmid (3 μ g/60 mm dish) using ICAfectinTM441 transfection reagent. Forty-eight hours post transfection, cells were either treated or left untreated with troglitazone for 16 h and the fluorescence intensity of GFP was measured for 10,000 cells using a FAC-Scan System (BD Biosciences, Caesarea, Israel), and the average intensity of the cell population was recorded for each treatment.

Protein carbonyl content

Protein carbonyl content was determined using the 2,4-Dinitrophenylhydrazine (DNPH) method as previously described.^{25,26}

Metabolic and biochemical analyses

Concentrations of very-long-chain fatty acids, phytanic and pristanic acid were determined as previously described by²⁷; concentrations of plasmalogens were determined as described.²⁷

Statistical analysis

To compare between groups we used the one-way analysis of variance (ANOVA) test. The Bonferroni correction for the significance level was applied when multiple pairwise comparisons were performed; a *P*-value of 5% or less was considered statistically significant.

Results

α -Syn expression affects catalase activity

Catalase activity was measured in samples of total proteins, extracted from whole brains, of A53T α -Syn or age-matched ntg control mice. Activity was determined using the Amplex Red assay kit (Molecular Probes) consisting of the ability of catalase to bind hydrogen peroxide (H₂O₂) at high affinity, with catalytic rate constant $k_0 > 10^7$ (mol/L)⁻¹ sec⁻¹ (Materials and Methods).²⁴ The relative activity is determined according to a standard curve using purified catalase, performed in parallel to the tested samples. The measured catalase activity was lower in both young and old (symptomatic) α -Syn mice than in control, age-matched mice. Specifically, the calculated catalase activity for young (1–2 months old) A53T α -Syn brains was 50.8 \pm 7.8 mU/mL per microgram protein. This activity was ~30.0% lower than the calculated activity for age-matched ntg control brains (64.4 \pm 7.4 mU/mL per microgram protein, Fig. 1A). In addition, the calculated catalase activity for old (10–12 months old) A53T α -Syn brains was 45.2 \pm 3.4 mU/mL per microgram protein, and 23.1% lower than in ntg control brains (59.9 \pm 7.8 mU/mL per microgram protein; Fig. 1A; *P* = 0.047, ANOVA). 3-Amino-1,2,4-triazole (3-AT), an inhibitor of catalase activity, was used to verify the specificity of the assay. In the presence of 20 mmol/L 3-AT, catalase activity was lowered by ~92–95%, suggesting that the activity measured represents catalase-specific activity.

The effect of α -Syn expression on catalase activity was similarly measured in cultured MN9D dopaminergic cells. Subconfluent cultures of cells, stably expressing either the mock plasmid or α -Syn, were tested for their catalase activity as above. Significantly lower catalase activity levels were calculated in MN9D clones expressing either wild-type or A53T α -Syn than in mock-expressing cells

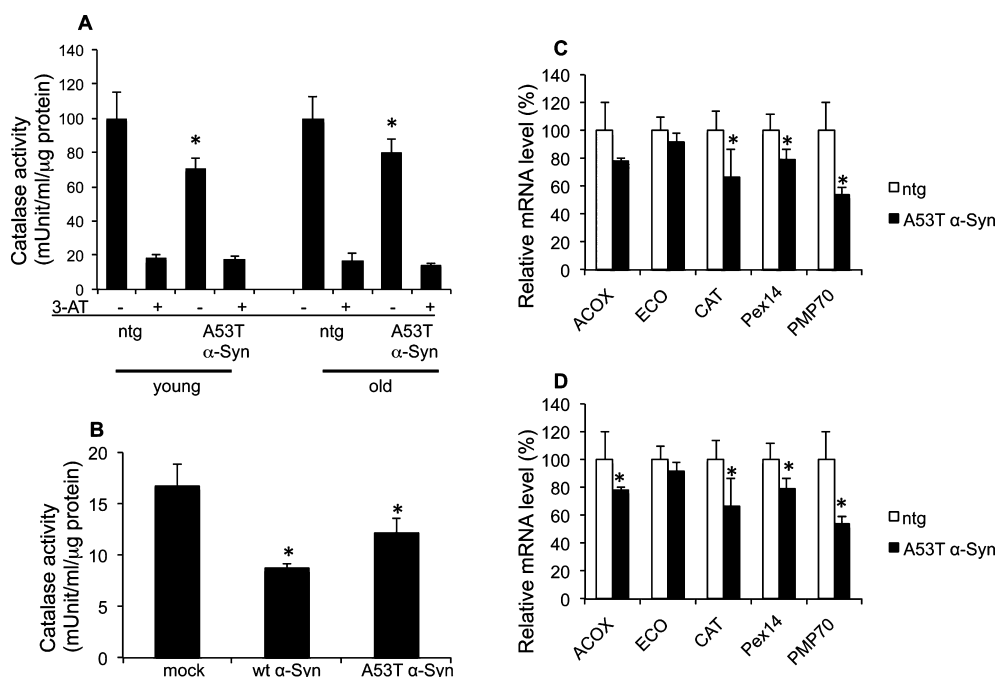


Figure 1. α -Syn inhibits catalase. (A) Catalase activity measured in protein extracts (20 ng) from young (1–2 month) or old (10–12 months), ntg or A53T α -Syn tg mouse brains by Amplex Red Catalase Assay kit (Molecular Probes) with or without the inhibitor, 3-AT (20 mmol/L); Mean \pm SD, $n = 4$ –6 mice in each age group; $P < 0.05$, ANOVA. (B) Catalase activity measured as in (A) in mock-transfected, wt, or A53T α -Syn overexpressing MN9D cells. Bars represent mean \pm SD of $n = 4$ independent measurements, each experiment consisted of two to three different clones. * P -value < 0.05 , ANOVA. (C) α -Syn affects the mRNA levels of genes transcriptionally regulated by PPARs. Total RNA was extracted from one mouse brain hemisphere ($n = 4$ –5 brains) of ntg and A53T α -Syn mouse brains at 1 month and at 8 months (D) and analyzed by real-time PCR with specific primers as indicated in the Materials and Methods. The results are normalized to the signal detected for each gene in the nontransgenic mouse brains. ACOX, acyl coenzyme A oxidase; ECO, enoyl coenzyme A hydratase; CAT, catalase; Pex14; and PMP70, peroxisome membrane protein 70. * $P < 0.05$, ANOVA.

(Fig. 1B). Specifically, the calculated catalase activity detected for the mock-transfected cells was 16.7 ± 2.2 mU/mL per microgram protein. Significantly lower activities were detected for human wild-type (8.7 ± 1.3 mU/mL per microgram protein) and A53T α -Syn (12.1 ± 2.5 mU/mL per microgram protein)-expressing cells ($n = 4$ independent measurements; consisted of two to four different clones; $P = 0.021$, ANOVA).

Lower catalase expression levels in A53T α -Syn mouse brains

The expression levels of catalase and additional genes, transcriptionally regulated by PPAR α , PPAR γ or their respective agonists, were determined by RT-PCR using specific primers designed for the following genes: acyl coenzyme A oxidase (ACOX)²⁸ and enoyl coenzyme A hydratase (ECO),²⁹ both enzymes involved in peroxisomal β -oxidation; peroxisome membrane protein 70 (PMP70)³⁰; and PEX14 (Pex14).³¹

The analyses were performed for young (1 month old) and old (10 months old) mice. Significantly lower mRNA

levels were detected for ACOX, catalase, Pex14 and PMP70 in brains of A53T α -Syn than in ntg controls. Yet, ECO mRNA levels did not differ between A53T and ntg brains in young or old mice. Specifically, the relative mRNA levels detected in young and old A53T α -Syn mice, normalized to the signals obtained for ntg mice, were 65.6 ± 6.46 and 78.1 ± 2.47 for ACOX; 64.7 ± 8.8 and 66.8 ± 20.1 for catalase; 72.6 ± 8.6 and 79.2 ± 7.06 for Pex14; 45.6 ± 7.2 and 54.3 ± 5.3 for PMP70 (Fig. 1C and D).

To determine catalase protein levels, peroxisome-enriched fractions were isolated from whole A53T α -Syn and ntg mouse brains (1–2 months old) and the relative levels of specific proteins were analyzed by quantitative Western blotting. Densitometric analysis of the Western blot revealed a significantly lower signal for catalase in peroxisome-enriched fractions obtained from A53T α -Syn ($81.4 \pm 4\%$) compared to ntg mouse brains (mean \pm SD, $n = 4$ brains; Fig. 2A). However, the levels of two other peroxisomal proteins, PMP70 and Pex14, tested in parallel, did not differ between transgenic and nontransgenic brains (Fig. 2A).

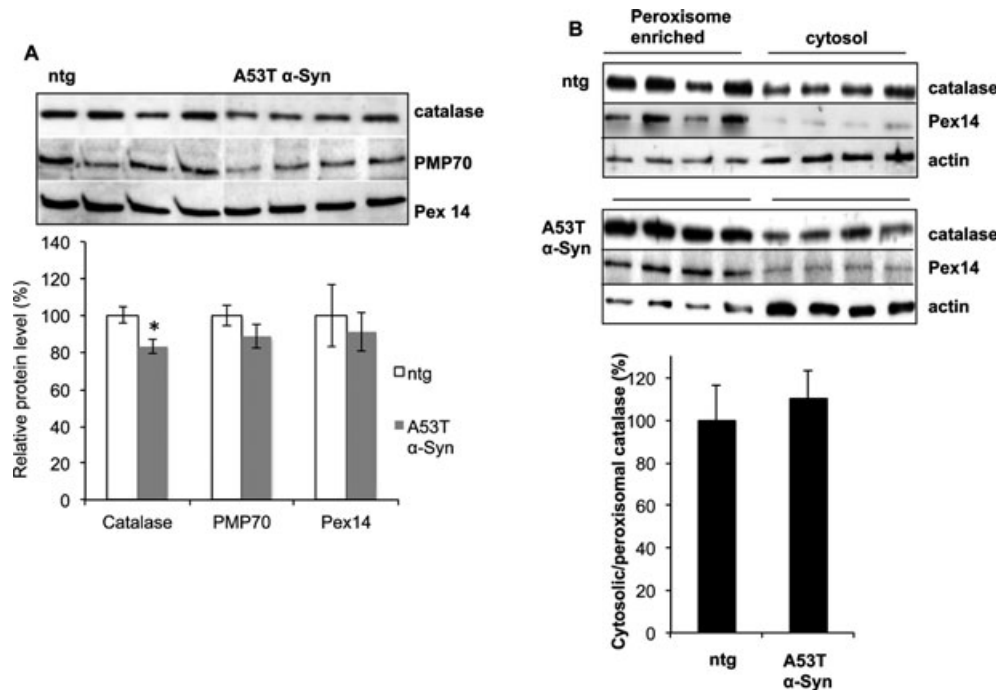


Figure 2. Lower catalase protein levels in peroxisome-enriched fractions of whole A53T α -Syn mouse brains. (A) Samples of peroxisome-enriched fractions (15 μ g protein) from nontransgenic and A53T α -Syn mouse brains (1–2 months old) analyzed by Western blotting using specific antibodies against catalase, PMP70 and Pex14. Bars represent mean \pm SD of $n = 4$ mouse brains. * $P < 0.05$; t -test. (B) The translocation of catalase to peroxisomes is unaffected in brains of A53T α -Syn mouse brains. Samples of peroxisome-enriched fractions and of corresponding cytosols analyzed as in (A), analyzed by Western blotting and reacted with anti-catalase and anti-Pex 14 antibodies. In addition to anti-actin antibody as a cytosolic protein. Bars represent mean \pm SD of $n = 4$ mouse brains in each genotype.

Next, we sought to find out whether α -Syn expression affects the translocation of catalase to peroxisomes. For this aim, we determined catalase and Pex 14 levels in peroxisome-enriched and in cytosolic fractions obtained from ntg and A53T α -Syn tg mouse brains. The blot was reacted also with anti-actin antibody, to determine the efficacy of fractionation. No differences in levels of cytosolic catalase or Pex-14 were detected. (Mean \pm SD, $n = 4$ brains, Fig. 2B), suggesting that the lower catalase protein level detected in peroxisome-enriched fractions does not represent affected translocation of catalase from cytosol to peroxisome.

To determine catalase protein levels specifically within neurons, we stained brain sections from young (1–2 months) and old (10–12 months), ntg and A53T α -Syn mice with anti-catalase or anti-Pex14 antibodies and quantified the signal that colocalized with the neuronal marker NeuN. In addition, we stained for endogenous α -Syn (ntg brains) and human transgenic α -Syn expression (A53T α -Syn brains) in contiguous brain sections. No signs of overt α -Syn accumulations were detected in the young A53T α -Syn brains, yet, in agreement with previous reports,^{13,16} α -Syn pathology was consistently observed in the brain stem and could also be detected in the cortex of old A53T α -Syn

mouse brains (Fig. 3A). A significantly lower signal for catalase (Fig. 3A–C) was detected within neurons in brain stem and cortex of young mice. That is, the relative catalase signals detected in young A53T α -Syn mice, normalized to the signals obtained for age-matched ntg mice set at 100%, were $68.5 \pm 13\%$ in the brain stem and $65.7 \pm 18\%$ in the cortex ($P < 0.05$, ANOVA). Similarly, lower catalase levels were also detected within neurons in old mouse brains. That is, $67.1 \pm 14.1\%$ in the brain stem and $60.7 \pm 20.2\%$ in the cortex. The lower signal of Pex14 reached significance in the brain stem of old mouse brains (70.1 ± 13.3 , Fig. 3B and C). The results represent mean \pm SD of $n = 4$ –7 mouse brains, with five to seven analyzed fields in each mouse brain. We concluded that catalase protein level is lower within neurons of A53T α -Syn mouse brains.

Unaffected catalase in young APP/PS1 mice modeling AD

To confirm that the effect of α -Syn on catalase expression and activity is specific to PD and not a result of general mechanisms of neurodegeneration, we used immunohistochemistry to assess catalase expression within neurons of APP/PS1 mice, which model the neurodegenerative

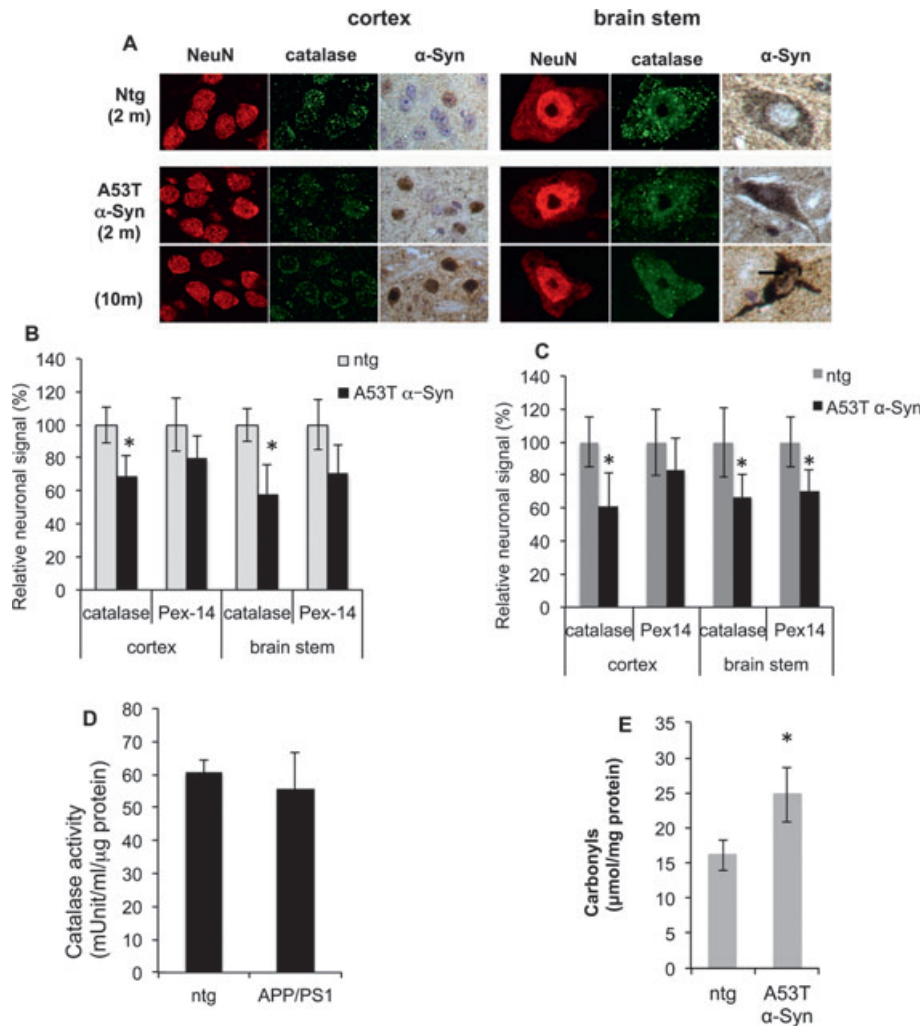


Figure 3. Lower catalase levels within neurons of A53T α -Syn mice. (A) Brain sections (5 μ m) from cortex or brain stem of nontransgenic (2 months old) and A53T α -Syn mice (2 and 10 months old), processed for immunohistochemistry and stained with anti-catalase, together with anti-NeuN for neuronal staining, or with anti-Syn-1 antibody for α -Syn expression in ntg brains or Syn-303 for A53T α -Syn brains. (B) Bars represent the signal colocalized with NeuN signal in young (1–2 months old) mouse brains. (C) As in (B) for old (10–12 months) mouse brains. Mean \pm SD of $n = 4$ –7 brains, with five to seven analyzed fields in each mouse brain. * $P < 0.05$ ANOVA. Bar = 10 μ m. (D) Catalase activity measured in brains of APP/PS1 mice and their control littermates as in Figure 1A. (E) The carbonyls content determined using 2,4-Dinitrophenylhydrazine in brains of A53T α -Syn and ntg mice. Mean \pm SD of $n = 4$ mice.

process characteristics of Alzheimer's disease (AD).³² Paraffin sections containing brain stem and cortex, from 2-month-old APP/PSEN1^{+/-} and their control ntg littermates were costained for catalase and NeuN as above. The catalase signal that colocalized with the signal obtained for NeuN was quantified. The results indicated no differences between APP/PSEN1 transgenic and control mice.

In addition, the enzymatic activity of catalase was determined in whole brain extracts of APP/PS1^{+/-} and

ntg littermates (2 months old). The results indicated no significant difference in catalase activity between the APP/PS1 and control littermates (Fig. 3D) catalase activity was 60.7 ± 3.6 mU/mL per microgram for APP/PS1^{+/-} and 57.1 ± 4.6 mU/mL per microgram protein for control mice. Of note, catalase activity determined, in parallel, in A53T α -Syn (1–2 months old) was significantly lower than ntg mouse brains (see Fig. 1A). We, therefore, concluded that catalase expression and activity are unaffected in this mouse model for AD.

Evidence for oxidative damage in brains of A53T α -Syn mice

The carbonyl content in proteins extracted from one hemisphere of ~8-month-old ntg and A53T α -Syn mouse brains was measured as an indicator for oxidative damage. The assay for protein carbonyls involves derivatization of the carbonyl groups with DNPH, which leads to the formation of a stable 2,4-dinitrophenyl (DNP) hydrazone product (see Materials and Methods). A significantly higher degree of protein carbonyls was detected in brains of A53T α -Syn than in ntg mouse brains. Specifically, we measured 24.8 ± 3.9 and 16.1 ± 2.25 μ mol DNP/mg protein for A53T α -Syn and ntg brains, respectively (Fig. 3E) mean \pm SD; $n = 4$ mouse brains; $P < 0.01$, t -test). On the basis of this result, we concluded that enhanced oxidative damage occurs in brains of A53T α -Syn mice.

α -Syn downregulates PPAR γ transcription activity

PPAR γ is involved in the regulation of catalase expression.¹⁴ To find out whether the lower catalase expression and activity levels, detected in brains of A53T α -Syn mice, may result from affected PPAR γ transcription activity, we utilized a PPAR γ reporter plasmid. The plasmid, consisting of a PPAR γ response element (PPRE) conjugated to

GFP, was transiently transfected into α -Syn overexpressing or mock-transfected clones of HeLa cells, and the GFP signal was measured by fluorescence-activated cell sorting (FACS) in live cells. The GFP signal, representing PPAR γ transcription activity, detected in α -Syn overexpressing cells was ~60% lower than that in mock-transfected clones. We next tested the effect of troglitazone, a specific PPAR γ agonist. Troglitazone further enhanced PPAR γ transcription activity, yet, its effect in control (mock) cells was stronger than in α -Syn-expressing cells (~50% and ~20% enhancement for control and α -Syn-expressing cells, respectively; Fig. 4A).

Since PPAR γ is a type II NR, constitutively bound to DNA, we next sought to find out whether the lower transcription activity detected in α -Syn-expressing cells may represent lower PPAR γ protein levels. For this aim, we quantified the PPAR γ signal obtained by Western blotting in clonal α -Syn overexpressing and mock-transfected HeLa cells. Two immuno-reactive bands were detected upon reacting the blot with an anti-PPAR γ antibody (Santa Cruz, sc-7273). According to their molecular mass, these bands represent PPAR γ 1 and PPAR γ 2, migrating at 54 and 57 kDa, respectively. Both PPAR γ 1 and PPAR γ 2 levels were lowered by α -Syn expression (Fig. 4B). Unlike the cultured cells, only low signals for PPAR γ were detected in whole brain extracts of young (2–3 months old) ntg and A53T α -Syn mice ($n = 4$ –5 brains). This signal did not differ between the two genotypes tested (Fig. 4C).

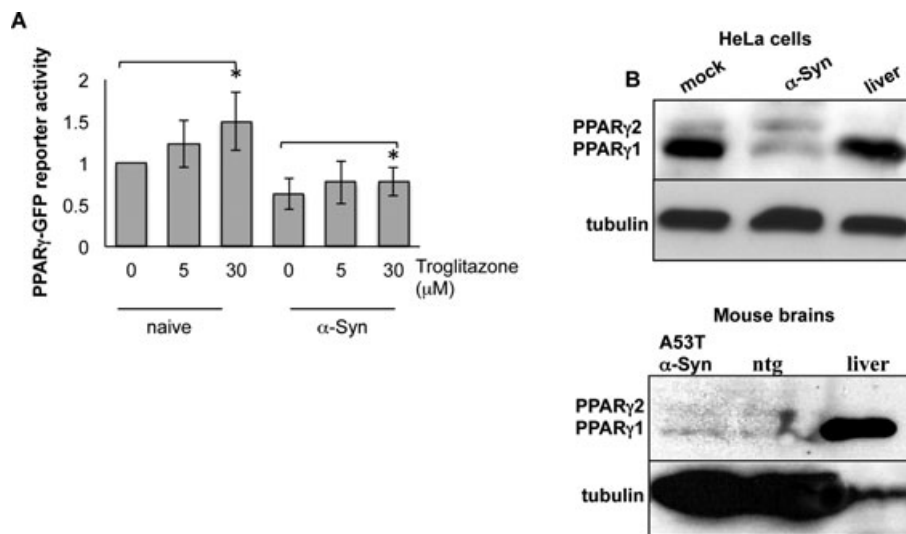


Figure 4. α -Syn affects PPAR γ activity. (A) Naive and α -Syn overexpressing HeLa cells were transfected with a PPAR γ -GFP reporter plasmid. Cells were treated with troglitazone (at 5 and 30 μ mol/L) or left untreated for 16 h and the GFP signal was measured by FACScan. The graph summarizes four experiments. $*P < 0.05$, ANOVA. (B) Protein samples of naive and α -Syn overexpressing cells (50 μ g protein), and (C) nontransgenic and A53T α -Syn mouse brains (120 μ g protein) and mouse liver (50 μ g protein), analyzed by Western blotting using an anti-PPAR γ antibody. The signal specificity was determined using a sample of mouse liver protein extract analyzed in parallel. A representative blot (out of three repeats).

Troglitazone and 9-cis-retinoic acid prevent the inhibitory effect of α -Syn on catalase activity

To verify the specific involvement of PPAR γ in catalase inhibition by α -Syn, we treated HeLa cells stably expressing α -Syn with troglitazone. Since PPAR γ heterodimerizes with RXR for transcription regulation, we also tested the effect of 9-cis-retinoic acid, an agonist of RXR. Cells were conditioned in medium containing either solvent only (DMSO), or the specific agonists, for 16 h. Catalase activity in control cells was 21.1 ± 4.2 mU/mL per microgram protein. Higher catalase activity was measured in cells treated with 5 μ mol/L troglitazone (29.06 ± 4.5 mU/mL per microgram protein) and significantly higher activity was measured with 30 μ mol/L troglitazone (37.2 ± 4.9 mU/mL per microgram protein). In addition, 9-cis-retinoic acid (5 μ mol/L) significantly enhanced catalase activity (52.6 ± 5.8 mU/mL per microgram protein, Fig. 5A). Importantly, following treatment with the agonist, catalase activity was highly similar in α -Syn-expressing and the control cells treated in parallel. In addition,

we confirmed the effect of troglitazone and 9-cis-retinoic acid on α -Syn oligomer levels¹³ (Fig. 5B). The levels of α -Syn oligomers, detected on a Western blot, were higher in cells treated with 5 μ mol/L troglitazone ($120 \pm 10.1\%$), 30 μ mol/L troglitazone (160 ± 11.0) or 5 μ mol/L 9-cis-retinoic acid ($171 \pm 12.3\%$).

Catalase activity is restored by PPAR α , PPAR γ and RXR

Next, we sought to examine the potential involvement of additional PPARs in catalase activity and its inhibition by α -Syn. For this aim, we transiently transfected HeLa cells with one of the following cDNAs: PPAR α , PPAR β/δ , PPAR γ 1, PPAR γ 2 or RXR or a mock plasmid. Each one of these transcription factors was cotransfected either with human α -Syn cDNA or without (α -Syn mock plasmid). Forty-eight hours later, RNA and protein were collected from the lysed cells. Transfection efficacy was determined by RT-PCR. Following transfection, the mRNA levels of α -Syn were higher by 20–75-fold in those cells transfected with an α -Syn cDNA. Accordingly, the mRNA levels of

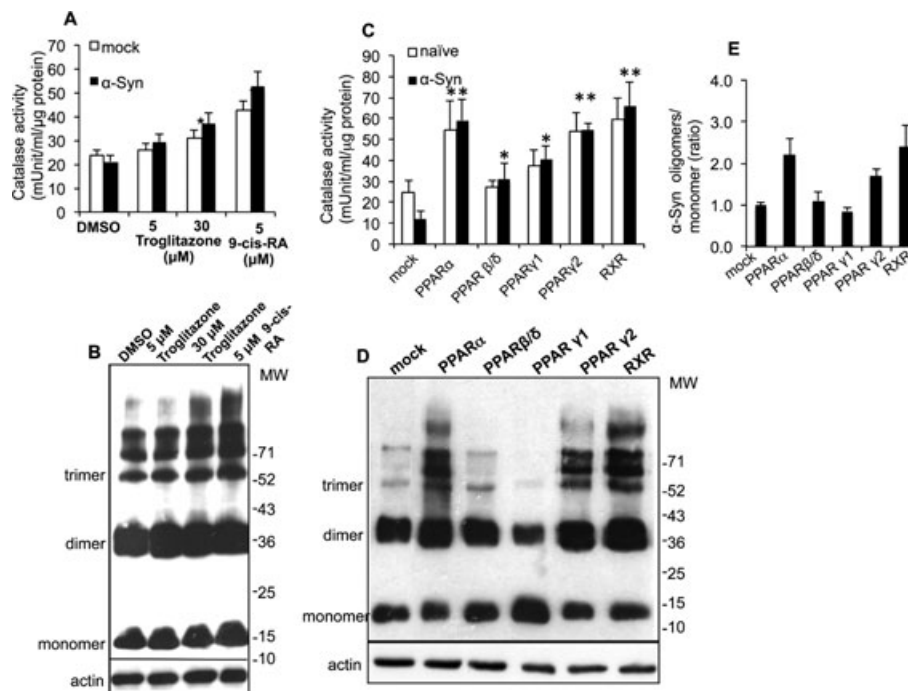


Figure 5. Catalase inhibition is eliminated by activation of PPAR α , PPAR γ or RXR. (A) Catalase activity measured in α -Syn-expressing-HeLa clonal cells, treated with troglitazone (5 and 30 μ mol/L) or 9-cis-retinoic acid (5 μ mol/L) for 16 h, determined as in Figure 1A. Mean of $n = 2$ –3 different clones \pm SD; * $P < 0.05$, one-way ANOVA. (B) Protein samples of high speed supernatant, of sister cultures treated in parallel and analyzed for α -Syn oligomers (see Materials and Methods) by Western blotting using anti- α -Syn antibody (LB-509). (C) Catalase activity measured in HeLa cells, transiently transfected with cDNA for the indicated nuclear receptors, together with (black bars) or without (white bars) α -Syn. Mean of $n = 4$ experiments \pm SD. * $P < 0.05$ ANOVA. (D) Western blot for α -Syn oligomers as described in (B). (E) Bar graph of Western blotting in (D) mean \pm SD of three repeats.

PPARα, PPARβ/δ, PPARγ1, PPARγ2, and RXR were elevated by 17–65-fold. The results suggested a variable effect for these factors on catalase activity (Fig. 5C). Specifically, the activity measured for cells without α-Syn overexpression, normalized to those cells transfected with a mock plasmid for the PPARs, was 24.9 ± 5.7 mU/mL per microgram protein. We found significantly higher 225–275% catalase activity in cells transfected with PPARα, PPARγ2 or RXR. Yet, PPARβ/δ and PPARγ1, although expressed at high levels (>50-folds), did not significantly affect catalase activity (Fig. 5C). Similar results were obtained in α-Syn-expressing cells, cotransfected either with PPARα, PPARγ2 or RXR. Catalase activity was significantly increased following the overexpression of these transcription factors to the levels detected without α-Syn (Fig. 5C). Specifically, normalizing the results to the activity measured for cells transfected with α-Syn and a mock plasmid for PPARs (16.1 ± 4.2 mU/mL per microgram protein), we found that catalase activity was increased by 329.9 ± 107.9% by PPARα, 257 ± 75.0% by PPARγ2, and 366 ± 68.2% by RXR. Moreover, overexpressing PPARγ1 and PPARβ/δ, also increased catalase activity in α-Syn overexpressing cells, that is, 193 ± 55.6% by PPARβ/δ and 234 ± 52% by PPARγ1.

The effect of PPARα, PPARβ/δ, PPARγ1, PPARγ2 or RXR on α-Syn oligomers was determined in parallel¹³ (Fig. 5D). The results confirmed the enhancing effect of PPARα, PPARγ2, and RXR overexpression on α-Syn oligomers specifically increasing α-Syn oligomers by 1.7–2.4-fold. No enhancing effect on α-Syn oligomers was observed with overexpression of PPARβ/δ or PPARγ1. These results, therefore, suggest an association between the levels of soluble α-Syn oligomers, detected on a Western blot following heat treatment³³ and catalase activity.

Peroxisome α-oxidation and β-oxidation are unaffected in brains of A53T α-Syn mice

On the basis of the above findings, suggesting that α-Syn interferes with the regulation of PPARγ activity and the

transcription levels of certain genes, we next sought to determine whether peroxisomes are affected in brains of A53T α-Syn mice. A gas chromatography–mass spectrometry (GC–MS) analysis was performed, measuring the levels of long-chain fatty acids. No significant differences were detected in levels of the saturated fatty acids, 20:0, 22:0, 24:0, or 26:0, indicating unaffected peroxisomal β-oxidation. This result is further supported by the C26:0/C22:0 ratio, which did not differ between ntg and A53T α-Syn mouse brains (Table 1). Similarly, no significant differences in levels of the branched chain phytanic acid or pristanic acid were found, indicating unaffected peroxisomal α-oxidation (Table 1). Of note, increases in certain polyunsaturated fatty acids (PUFA) were detected in brains of A53T α-Syn mice. Specifically, significantly higher levels of 20:5 ω3 (*P* = 0.001); Docosahexaenoic acid 22:6 ω3 (*P* = 0.02); 22:4 ω6 (*P* = 0.02); and 22:5 ω6 (*P* = 0.044; one-way ANOVA, with *n* = 6 mouse brains in each group). This result is consistent with our previous report,³⁴ yet, does not support peroxisomal dysfunction.

Interestingly, GC–MS revealed significantly higher levels of plasmalogens in brains of A53T α-Syn mice. Specifically, alkenylacyl-glycerophospholipids levels, expressed as the percentages of dimethylacetal (DMA) derivatives of either C16:0 or C18:0 to the corresponding saturated fatty acid, were higher in brains of A53T α-Syn mice (Table 1). The higher plasmalogen levels in brains of A53T α-Syn mice, may suggest an altered balance between synthesis and degradation. Together, the biochemical analyses indicate that some peroxisomal activities are altered, with no indication for peroxisome dysfunction.

Discussion

We report an inhibitory effect for α-Syn expression on catalase mRNA and protein levels, resulting in lower catalase activity and accumulation of oxidative injury. Utilizing the A53T α-Syn transgenic mouse as a model for PD and the related synucleinopathies,^{16,35} we show that the effect on catalase is age-independent and observed in brains of young as well as in aged, symptomatic mice. The finding that catalase expression and activity are unaffected in brains of mice modeling AD provides evidence for the specificity of the α-Syn effect. Moreover, we show that the mechanism through which α-Syn inhibits catalase involves inhibition of PPARγ, a NR that controls catalase expression. Activating PPARα, PPARγ2 or RXR restored catalase activity, and activation of PPARβ/δ and PPARγ1, enhanced catalase activity. The results suggest that α-Syn interferes with the regulation of catalase expression and activity, and provides novel insights into the causes of oxidative stress observed in brains of patients with PD.

Table 1. Analysis of specific peroxisomal metabolites.

	Nontransgenic	A53T α-Syn	<i>P</i> -value
C26:0/C22:0	0.027 ± 0.005	0.022 ± 0.003	0.536
Phytanic acid (nmol/mg protein)	0.019 ± 0.016	0.01 ± 0.006	0.226
Pristanic acid (nmol/mg protein)	0.003 ± 0.002	0.007 ± 0.004	0.079
C16:0 DMA/C16:0	8.5 ± 0.30	9.08 ± 0.40	0.019*
C18:0 DMA/C18:0	15.92 ± 0.35	16.97 ± 0.54	0.003*

Mean ± SD of six mouse brains in each genotype.

**P*-value <5%, ANOVA.

We report two observations related to the activation of PPAR α , PPAR γ 2, and RXR. The first is upregulation of catalase activity and the second is a conformational alteration in α -Syn, presented as soluble α -Syn oligomers on the Western blot, following a heat treatment. These observations raised the question whether specific soluble α -Syn forms, potentially oligomeric forms, regulate catalase activity. Importantly, catalase expression and activity were similarly affected in young, healthy, mice and in old, symptomatic, mice. In addition, the effect on catalase activity was similarly lower in human wt and A53T α -Syn-expressing cells. Together, these results suggest that the effects described herein on catalase expression and activity are not related to α -Syn pathogenesis. These observations stress the importance of identifying the actual physiological form(s) of α -Syn and distinguish those from the pathogenic α -Syn forms. Of note, many of the reported treatments that enhance α -Syn oligomerization are also known for their effect to activate NRs and specifically, PPARs, including, DHA,^{13,36,37} oxidative and nitrosative stress,³⁸ and inflammatory responses.³⁸

There are 48 known human NRs that form a complex network of gene activation pathways. A high degree of overlapping between NR-activated pathways complicates investigative efforts to understand and identify their distinct effects. Although it has been well established that NRs control diverse mechanisms, including development, inflammation, reproduction, and metabolism, their role in neurodegeneration is less clear. Nevertheless, the involvement of NRs in synucleinopathies was highlighted when rare mutations in the orphan NR, Nurr1, were associated with familial late-onset PD^{39,40} providing genetic evidence for the involvement of these transcription regulators in PD. We show that α -Syn inhibition of catalase activity can be eliminated by activation of certain NRs. It is plausible that activation of specific NRs may affect the fine balance of this complex network. A comprehensive study is needed to define the specific NR's pathways that are involved in α -Syn regulation of catalase.

Lower catalase activity results in elevated levels of its substrate, hydrogen peroxide (H₂O₂), and is associated with aging and age-related diseases.⁴¹ At low concentrations, H₂O₂ is a critical signaling molecule, involved in carbohydrate metabolism, cell proliferation and apoptosis.⁴² At high concentrations, H₂O₂ is toxic, mainly because it can be easily converted into ROS like superoxide anion (O₂^{•-}) and hydroxyl radical (•OH). Similar to H₂O₂, ROS have been shown to play a role both in pathogenic and physiological mechanisms and in aging.^{43,44} Hydroxyl radicals may attack the double bonds in fatty acyl, primarily n-6 PUFA, side chains of phospholipids, forming 4-hydroxynonenal (4-HNE) oxidation products. 4-HNE is an abundant reactive carbonyl molecule produced in

neurodegeneration.⁴⁵ A chemical reaction of 4-HNE with lysine, histidine or cysteine residues⁴⁶ results in protein carbonylation. Thus, the content of protein carbonyl is the most general and well-used biomarker of oxidative damage. Nevertheless, brain levels of catalase are considerably lower than its levels in the liver. Therefore, it is expected that for the removal of brain H₂O₂, additional peroxidases and peroxiredoxins are involved. In this study, we focused on the effect of α -Syn on catalase, yet, it will be interesting to find out whether it might also affect additional enzymes involved in the detoxification of H₂O₂.

Catalase activity and expression level were compared between young (2 months old), healthy mice, modeling either AD (APP/PS1 tg) or PD (A53T α -Syn tg). Both AD and PD neurodegenerations strongly associate with aging. The free radical theory of aging postulates that aging results from damage caused by the accumulation of ROS.⁴⁷ However, this theory is a matter of debate. More recent data, suggest that ROS are actually required for lifespan extension^{48,49} and challenge this theory. Importantly, affected catalase expression, stability or localization to peroxisomes strongly correlates with aging and age-related diseases.⁵⁰ In accord, overexpression of human catalase in the mitochondria of mice extended lifespan.⁵¹ Together, these evidences support an association between catalase and mechanisms of aging. To distinguish between α -Syn effect and general aging effect on catalase activity, we compared the young mice, which show no evidence (behavioral or pathological) for the occurrence of the disease.^{13,16} The results showing an effect for α -Syn on catalase activity at the young A53T α -Syn mice suggest that α -Syn interferes with catalase activity independently from aging or age-related neurodegenerations.

The mitochondria are a major source of ROS, generated through the in oxygen by the respiratory chain and oxidative phosphorylation system. Defects in the respiratory chain function, whether inherent or induced by toxins, cause an excess of free radical production.⁵² Indeed mitochondrial dysfunction is critically implicated in the pathogenesis of PD. Several lines of evidence support a role for mitochondrial dysfunction in the pathogenesis of PD. Familial forms of PD involve specific mutations in mitochondrial genes; mitochondrial complex I activity is inhibited in postmortem PD brains; and several mitochondrial toxins were shown to cause PD (recently reviewed by⁵²). While the role of mitochondria in the pathogenesis of PD is being extensively investigated, the role of peroxisomes, as key players in PD and relevant age-dependent neurodegenerations, is still unknown. Here we show evidence linking α -Syn expression and dysfunction of catalase, an enzyme residing primarily in peroxisomes. Our results point at catalase inhibition as a potential source for the oxidative stress that characterizes

the pathogenic mechanisms leading to PD. It is estimated that ~35% of the cellular H₂O₂ is produced by peroxisomes.⁵³ This figure exemplifies the importance of elucidating the potential role played by peroxisomes in neurodegeneration and, particularly, in PD.

Oxidative damage has been implicated in the pathogenesis of α -Syn in the synucleinopathies. Specifically, nitrosylation of α -Syn at tyrosine residues, forming 3-nitro-tyrosine, was detected at high levels in pathogenic signatures of this group of neurodegenerations.^{54,55} Furthermore, nitrosylation of tyrosine residues on α -Syn protein results in stabilization of its oligomers.^{56,57} It is plausible that the lower catalase activity described in this study may lead to increased H₂O₂ levels and, subsequently, to higher ROS production, which acts to modify proteins in the form of 3-nitro-tyrosine. Nitric oxide is produced in vivo by the inflammatory stimuli-induced nitric oxide synthase (iNOS) and by the constitutive forms of this enzyme (eNOS and nNOS). Nitric oxide pathology depends on the formation of reactive nitrogen species (RNS) such as peroxynitrite anion (ONOO⁻) and nitrogen dioxide (\bullet NO). The formation of RNS from nitric oxide requires the presence of oxidants such as superoxide radicals (O₂^{•-}), H₂O₂, and transition metal centers (reviewed by⁵⁸), which can convert native tyrosine residues in proteins into 3-nitro-tyrosine. Therefore, the lower catalase activity described in this study may explain the accumulation of nitrosylated α -Syn in pathogenic conditions.

PPAR γ is a ligand-activated transcription factor belonging to the NR super family. It preferentially dimerizes with RXR to control the transcription of various genes involved in cellular energy metabolism. Among the activating ligands identified for PPAR γ are PUFAs such as linolenic acid (18:3), eicosapentaenoic acid (20:5), and docosahexaenoic acid (22:6); the naturally occurring oxidized fatty acid (*S*)-hydroxy-octadecadienoic acid (HODE, 18:2); and the fatty acid metabolites prostaglandin J₂ (PGJ₂) and eicosanoids such as hydroxyeicosatetraenoic acids (HETEs). Importantly, growing evidence suggests that α -Syn expression and pathology associate with alterations in levels of certain PUFAs and their metabolites.^{34,59–62} It is, therefore, possible that α -Syn affects PPAR γ transcription activity by affecting the availability or local concentrations of specific PPAR γ -activating ligands.

A recent meta-analysis of 17 independent genome-wide gene expression microarray studies has identified several gene sets that are strongly associated with PD.⁶³ The gene sets with the strongest association indicated reduced expression of genes that are involved in energy metabolism, primarily glucose metabolism, and nuclear genes encoding subunits of the electron transport chain proteins found in mitochondria. Genes whose expression is controlled by

PPAR γ -coactivator-1 α (PGC-1 α) are underexpressed in patients with PD. An additional study has reported that when localized to the nucleus, α -Syn binds the PGC1 α promoter and inhibits its activity, resulting in lower PGC1 α mRNA levels.⁶⁴ Accordingly, activation of PGC-1 α rescue dopaminergic neurons from α -Syn-induced toxicity.^{63,64} Together these studies may suggest that α -Syn inhibits catalase activity through its effect on PGC1 α activity.

A biochemical analysis was performed to determine levels of specific peroxisome metabolites in brains of ntg and A53T α -Syn mice. The analysis revealed similar levels of metabolites generated in the pathways of peroxisomal α - and β -oxidation suggesting that the activity in these pathways is unaffected. The biosynthetic pathway of plasmalogens involves several enzymatic steps performed in peroxisomes and the endoplasmic reticulum.^{65,66} Deficiency in the biogenesis of peroxisomes or one of the two peroxisomal enzymes involved in plasmalogen biosynthesis, that is, glyceronephosphate O-acyltransferase and alkylglycerone phosphate synthase, leads to reduction or complete elimination of these ether-phospholipids.^{67–70} However, higher plasmalogen levels were detected in brains of A53T α -Syn mice. Together, the biochemical analysis indicated that peroxisome activity is unaffected in mature A53T α -Syn mice. The significantly higher plasmalogen levels detected in A53T α -Syn mice may represent a specific α -Syn-dependent effect on brain lipids that is potentially independent of their biosynthesis.

In a previous study, we showed that the accumulation of α -Syn in oligomeric and aggregated forms is enhanced in brains of mice modeling peroxisome biogenesis disorders (PBD).⁷¹ Our results suggested that in brains of PBD models, α -Syn assemblies are affected by the altered lipid metabolism and, specifically, by the accumulation of long-chain, n-6 polyunsaturated fatty acids. Interestingly, we found no direct evidence for an association between oxidative stress and pathogenic α -Syn accumulation in the PBD models. In this study, we have studied the potential effects of α -Syn expression on peroxisome gene expression and activity. These findings suggest that α -Syn affects catalase through its effect on PPAR γ activity. Since PPAR γ activity is regulated by energy- and, specifically, lipid metabolites, it is possible that α -Syn is involved in a metabolic loop, where its assembly in specific forms is affected by lipid metabolites and, in response, affects brain lipid and energy metabolism.

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Conflict of Interest

E. Y., H. K., J. G., and R. S.: employer: Hebrew University, grant/grants pending. W. K. and R. J. A. W., employer: University of Amsterdam, consultancy.

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