Phosphorylation of α-Synuclein Protein at Ser-129 Reduces Neuronal Dysfunction by Lowering Its Membrane Binding Property in Caenorhabditis elegans*§

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Background: The pathogenic role of phosphorylation of α-synuclein in Parkinson disease remains unclear.

Results: Ser-129-substituted α-synuclein was expressed in transgenic C. elegans. Nonphosphorylatable (S129A) α-synuclein showed severe defects. The level of membrane-bound α-synuclein was increased in S129A-α-synuclein.

Conclusion: Ser-129 phosphorylation of α-synuclein attenuates neuronal dysfunction and downstream stress response by lowering the membrane binding property.

Significance: Phosphorylation of α-synuclein is protective against neurotoxicity.

α-Synuclein is causative for autosomal dominant familial Parkinson disease and dementia with Lewy bodies, and the phosphorylation of α-synuclein at residue Ser-129 is a key posttranslational modification detected in Parkinson disease/dementia with Lewy bodies lesions. However, the role of Ser-129 phosphorylation on the pathogenesis of Parkinson disease/dementia with Lewy bodies remains unclear. Here we investigated the neurotoxicity of Ser-129-substituted α-synuclein in the transgenic Caenorhabditis elegans (Tg worm) model of synucleinopathy. Tg worms pan-neuronally overexpressing nonphosphorylatable (S129A) α-synuclein showed severe defects including motor dysfunction, growth retardation, and synaptic abnormalities. In contrast, Tg worms expressing phosphorylation mimic (S129D) α-synuclein exhibited nearly normal phenotypes. Biochemical fractionation revealed that the level of membrane-bound α-synuclein was significantly increased in S129A-α-synuclein Tg worms, whereas S129D- and as well as A30P-α-synuclein displayed lower membrane binding properties. Furthermore, A30P/S129A double mutant α-synuclein did not cause neuronal dysfunction and displayed low membrane binding property. In human neuroblastoma SH-SY5Y cells, localization of S129A-α-synuclein to membranes was significantly increased. Finally, gene expression profiling of S129A-Tg worms revealed a dramatic up-regulation of Daf-16/FOXO pathway genes, which likely act against the dysfunction caused by S129A-α-synuclein. These results imply a role of Ser-129 phosphorylation of α-synuclein in the attenuation of α-synuclein-induced neuronal dysfunction and downstream stress response by lowering the membrane binding property.

Mutations or multiplications of α-synuclein gene have been identified as a cause of rare autosomal dominant forms of familial Parkinson disease and dementia with Lewy bodies. To date, three missense mutations (A53T, A30P, E46K), triplication, and duplication were reported (1–6). In addition, polymorphisms in the promoter region (7, 8) and untranslated region (9) of α-synuclein gene are also reported as a risk of Parkinson disease or dementia with Lewy bodies, and recent genome-wide association studies also identified α-synuclein gene as one of the major risk loci for sporadic Parkinson disease (10, 11). The formation of Lewy bodies, which contain α-synuclein as a major component (12, 13), is the hallmark of neuropathological change in Parkinson disease and dementia with Lewy bodies, leading to the collective nomenclature of α-synucleinopathy. α-Synuclein deposited in α-synucleinopathy lesions is characteristically phosphorylated at Ser-129 (14). Thus, the understanding of the significance of the deposition and phosphorylation of α-synuclein in neurons may open up a way to the development of novel therapeutics for α-synucleinopathy.

α-Synuclein is a largely cytosolic protein, whereas a portion of α-synuclein is thought to be associated with cellular membranes through the N-terminal KETKEGV repeats (15–18); this association has been reported to be disrupted by the A30P mutation (16, 19, 20). Although the physiological significance of the interaction of α-synuclein to membranes remains unclear, α-synuclein knock-out mice exhibited defects in paired pulse inhibition of dopaminergic nerve terminals (21), which is accompanied by an overall reduction in size of the distal reserve pool of synaptic vesicles (22), and an antisense RNA knockdown of α-synuclein in hippocampal slice cultures similarly yields a reduction in the distal reserve synaptic vesicle pool (23). These studies implicate α-synuclein in the regulation of mobilization or maintenance of synaptic vesicles at the presynaptic terminals. Moreover, an in vitro study suggested that the binding of α-synuclein to artificial liposomes was significantly decreased by Ser-129 phosphorylation by G protein-coupled receptor kinase 5 (24), suggesting a role of Ser-129 phos-
Phosphorylation on the regulation of membrane/vesicle binding of α-synuclein. However, it remains unknown how the membrane binding and phosphorylation of α-synuclein in vivo impact the function and neurotoxicity of α-synuclein.

To investigate the effect of Ser-129 phosphorylation on α-synuclein neurotoxicity in vivo, a series of transgenic (Tg)2 animals that overexpress mutant α-synuclein replaced at Ser-129 with alanine (S129A), a nonphosphorylatable form, or aspartate (S129D), a phosphorylation mimic form, have been generated. Two groups have reported that overexpression of recombinant adeno-associated virus-mediated S129A-α-synuclein in rat substantia nigra caused prominent loss of dopaminergic neurons, whereas that of S129D-α-synuclein displayed much milder toxicity (25, 26). Conversely, another rat model exhibited no differences in toxicities among wild-type (WT) and Ser-129 mutant α-synuclein expressed by recombinant adeno-associated virus (27), and S129D-α-synuclein showed more severe toxicity when compared with S129A-α-synuclein in Tg Drosophila (28). Thus, there remain discrepancies in the neurotoxicity of Ser-129 mutant α-synuclein among animal models, and the mechanisms underlying the toxicity are still unclear. Previously, we have generated Tg Caenorhabditis elegans overexpressing human WT and pathogenic mutant (A53T, A30P) α-synuclein in a pan-neuronal fashion (29). Although simple overexpression of WT or mutant α-synuclein alone was not sufficient to cause neurotoxicity, we invariably detected Ser-129 phosphorylation of α-synuclein in these Tg worms. Thus, the role of phosphorylation at Ser-129 on α-synuclein neurotoxicity also remained elusive in the C. elegans model.

Here we report the effect of Ser-129 substitution of α-synuclein in C. elegans neurons. S129A-α-synuclein caused severe phenotypic defects and increased binding to membranes, whereas S129D as well as A30P/S129A-α-synuclein showed few defects and decreased binding to membranes. Overexpression of S129A-α-synuclein resulted in the up-regulation of DAF-16/Forkhead box O (FOXO) target genes involved in stress response, and genetic deletion of daf-16 dramatically suppressed the phenotypes of S129A-Tg worms. These results suggest a novel link among α-synuclein phosphorylation, membrane association, and stress response in the mechanism of α-synuclein-mediated neuronal dysfunction.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—punc-syn2::α-synuclein constructs including A53T and A30P mutants were previously described (29). cDNAs of S129A, S129D, and A30P/S129A α-synuclein were generated by the overlapping PCR mutagenesis strategy. All constructs were sequenced using Thermo Sequenase™ (Amersham Biosciences) on an automated sequencer (LI-COR, Lincoln, NE).

**C. elegans Protocols**—Nematodes were handled by standard methods (30). N2 is the wild-type strain. Strains carrying daf-16(mu86) and juls1(Punc-25::snb-1::gfp) were obtained from the Caenorhabditis Genetics Center (University of Minnesota, St. Paul, MN). Worms were grown on nematode growth medium agar plates inoculated with Escherichia coli strain OP50 at 20 °C. For the generation of Tg worms pan-neuronally overexpressing Ser-129 mutant α-synuclein, plasmid DNAs encoding each mutant-α-synuclein were mixed with a marker plasmid Punc-S1::EGFP (enhanced green fluorescent protein), and the mixtures (200 μg/ml each) were co-injected into the gonads of young adult N2 worms. Transgenic progeny carrying α-synuclein as extrachromosomal arrays were selected on the basis of the expression of markers. Transgenes were integrated into chromosomes by ultraviolet irradiation, and each isolated animal was outcrossed eight times. Worms carrying both S129A-α-synuclein transgene and daf-16(mu86) were generated by mating α-synuclein Tg worm with daf-16(mu86) mutant, and the genotyping was performed by PCR using following set of primers: 5’-TTCTTCACTCGCCCTTCATCA-3’ and 5’-GGCGAGATTGTTCAGTTGA-3’ for deleting deletion and 5’-TTCCCTTCACATTCACTTCC-3’ and 5’-CCCCATGGTTGTTCTCCTCT-3’ for detecting the absence of deletion. Worms carrying α-synuclein and Punc-25::snb-1::gfp were generated by injecting plasmid encoding α-synuclein into juls1(Punc-25::snb-1::gfp). Synchronized culture of worms was generated by isolation of eggs via the hypochlorite method as described (31).

**Immunohistochemistry**—Preparation of paraffin-embedded worm sections and immunostaining were performed as described (29). α-Synuclein was detected with a mouse monoclonal antibody Syn211 (32), and α-synuclein phosphorylated at serine 129 was detected with a mouse monoclonal antibody pSyn#64 (33).

**Western Blot**—Worms were washed three times with M9 buffer (22 mm KH₂PO₄, 22 mm Na₂HPO₄, 85 mm NaCl, 1 mm MgSO₄), and worm pellets were frozen at −80 °C. Extracts were prepared by resuspending frozen pellets in SDS sample buffer and sonication. The sonicates were centrifuged at 10,000 × g for 5 min, and the supernatants were collected. 30 μg of protein per each sample was separated by SDS-PAGE followed by immunoblotting as described previously (29). α-Synuclein was detected with an antibody LS509 (13), and α-tubulin was detected with an antibody DM1A (Sigma).

**Detergent Fractionation Analysis**—Worms were washed three times with M9 buffer and collected as an ~100-μl pellet, and the pellet was frozen and stored at −80 °C. The pellets were homogenized in 50 mm Tris-HCl buffer at pH 7.5 with Complete protease inhibitor mixture (Roche Applied Science) by brief sonication, and the sonicates were centrifuged two times at 1000 × g for 5 min to remove debris of worm tissue. The supernatant was then ultracentrifuged at 350,000 × g for 15 min, and the supernatant was collected. 30 μg of protein per each sample was separated by SDS-PAGE followed by immunoblotting as described previously (29). α-Synuclein was detected with an antibody LS509 (13), and α-tubulin was detected with an antibody DM1A (Sigma).

2 The abbreviations used are: Tg, transgenic; EGFP, enhanced green fluorescent protein; SynWT-TG, WT-α-synuclein-Tg; FOXO, Forkhead box O.
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Phenotypic Analysis—A liquid thrashing assay was performed by placing individual young adult worms into the isotonic M9 buffer, and after 30 s, counting the frequency of body bends per 1 min. Growth rate was examined by measuring the body length of the slide-mounted worms at each stage under a microscope.

Fluorescence Microscopy—Worms were anesthetized by placing them in a drop of 50 mM sodium azide in M9 buffer, which was put on the solidified pads of 4% agarose laid on the slides. After the addition of a coverslip, worms were examined with an Axio Observer.Z1 microscope equipped with the AxioVision 4.6 software (Carl Zeiss).

Immunocytochemistry Using SH-SYSY Cells—Human neuroblastoma SH-SYSY cell line was maintained in a mixture of F12 and Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37 °C in a 5% CO2 atmosphere. Transfection was performed with Lipofectamine 2000 reagent (Invitrogen) following the manufacturer’s instructions. Digitonin permeabilization of cells was carried out essentially as described previously (34). Twenty-four hours after transfection, the cells were permeabilized for 3 min in an ice-cold buffer (12.5 mM HEPES—KOH, pH 7.4, 50 mM PIPES—KOH, pH 6.9, 1 mM MgSO4, and 4 mM EGTA—KOH) containing 40 μM digitonin (Sigma). After permeabilization, cells were washed four times briefly in PBS. The cells were fixed in the fixation buffer (4% paraformaldehyde, 4% sucrose in PBS) and blocked and permeabilized in the blocking buffer (3% bovine serum albumin, 0.1% Triton X-100 in PBS). Immunolabeling with anti-bovine serum albumin, 0.1% Triton X-100 in PBS). Immunolabeling with anti-α-synuclein LB509 or anti-phosphorylated α-synuclein pSer129 antibodies was visualized by an indirect immunofluorescence method using Alexa Fluor-conjugated secondary antibodies (Invitrogen), and the nuclei were stained with SYTOX Orange (Invitrogen). Fluorescence images were acquired on a confocal laser-scanning microscope (LSM510 Zeiss) using a fixed laser power and detector gain for all samples and quantified using the ImageJ software.

DNA Microarray—Synchronized young adult worms of N2, WT-α-synuclein-Tg (synWT-Tg), A53T-Tg, and S129A-Tg genotypes were washed three times with M9 buffer and collected as a 100-μl worm pellet, and total RNAs were isolated using the ISOGEN reagent (Nippon Gene). Subsequent reverse transcription and cRNA synthesis were performed using One-Step target labelling and control reagents (Affymetrix), and the fragmented biotinylated cRNAs were hybridized to the Affymetrix C. elegans genome array, which represents 22,500 transcripts from C. elegans. Signal intensity of each strain was normalized by the median value of the transcripts, and genes whose expression levels were up-regulated or down-regulated more than 1.5-fold when compared with N2 were selected.

Real-time RT-PCR Analysis—Total RNA was isolated from 30 μl of worm pellet using the ISOGEN reagent (Nippon Gene), and cDNA synthesis was performed from 1 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time RT-PCR analysis was performed using the LightCycler 480 system (Roche Applied Science). Primer sequences for real-time PCR are as follows: 5′-AACCTGTCTGAGCGGTTCT-3′ and 5′-TCCATGAAGTCTCACTAATCTCA-3′ for mlt-1, 5′-GGAGGCTGGTCCTCAGGAAA-3′ and 5′-ACATGAAACCGGCTTATCTC-3′ for dod-3, and 5′-CCCATCAGGAGAGTATCCTG-3′ and 5′-ACATGGCTGGTATTGAAG-3′ for act-2. Expression levels of genes were normalized by the level of act-2 in each strain.

Statistics—All quantitative data presented are expressed as the mean ± S.E. The results were statistically evaluated for significance by using one-way analysis of variance with Fisher’s protected least significant difference test. Differences were considered significant when p < 0.01 unless otherwise noted.

RESULTS

Generation of Tg C. elegans Overexpressing Ser-129 Mutant α-Synuclein—We have previously reported transgenic worms overexpressing human α-synuclein (WT, A53T, A30P) in a pan-neuronal fashion under the control of unc-51 promoter (29). Although these Tg worms did not show any severe neurological defects, phosphorylation of α-synuclein at Ser-129, a characteristic posttranslational modification in synucleinopathy lesions, was invariably detected. To examine the role of Ser-129 phosphorylation in α-synuclein neurotoxicity, we generated Tg worms pan-neuronally overexpressing S129A- or S129D-α-synuclein, representing nonphosphorylatable or phosphorylation mimic α-synuclein, respectively. The plasmid harboring S129A- or S129D-α-synuclein downstream of the unc-51 promoter (Fig. 1A) was microinjected into wild-type N2 worms together with Punc-51:EGFP (enhanced green fluorescent protein) as a transgenic marker, and chromosomally integrated strains were obtained. Western blot analysis revealed that both S129A-α-synuclein and S129D-α-synuclein were abundantly expressed, at similar levels to previously generated Tg worms expressing WT α-synuclein (Fig. 1B). Furthermore, immunohistochemical analysis of paraffin-embedded sections of Tg worms revealed that both S129A-α-synuclein and S129D-α-synuclein were mainly expressed in neurons including nerve ring and ventral nerve cord (Fig. 1C). We also confirmed that these Tg worms do not react with the antibody against Ser-129-phosphorylated α-synuclein (Fig. 1D).

S129A-α-Synuclein Tg Worms Showed Severe Motor Dysfunction, Growth Retardation, and Synaptic Abnormality—We found that Tg worms expressing S129A-α-synuclein (S129A-Tg) were obviously defective in growth rate and motor function, whereas Tg worms expressing S129D-α-synuclein (S129D-Tg) appeared nearly normal (Fig. 2A). A characteristic feature of S129A-Tg worms was the coiler phenotype, representing motor dysfunction (Fig. 2A, inset). To quantitatively evaluate the growth rate, we measured the length of individual worms over time after hatch and found that two independent lines of S129A-Tg worms were significantly defective in growth rate when compared with other strains including synWT-Tg and S129D-Tg worms (Fig. 2B). Furthermore, we assessed the motor function of each strain at day 1 (L2 larva), day 3 (young adult), and day 8 by counting the frequency of body bends in the isotonic liquid. We found that two independent S129A-Tg strains showed strikingly severe motor defects throughout development and aging, suggesting constant neuronal dysfunc-
Overall morphology of S129A-Tg worms, which grew up to adulthood, was also abnormal, and a majority of these worms showed a severe egg-laying defect (Fig. 2D). In addition, we examined the superficial morphologies of S129A-Tg worms by differential interference microscope and found that abnormal swellings of intestine and defective morphogenesis of gonad, associated with tissue vacuolation in the hypodermis of the head, were observed (supplemental Fig. 1, and data not shown). We also assessed the possible occurrence of morphological changes of neurons expressing S129A-α-synuclein by observing the fluorescence of EGFP, which was co-expressed as a marker. However, no apparent abnormalities, e.g. disappearance of neurites or nerve cell body, were observed, and TUNEL staining was negative in S129A-Tg worms (data not shown).

We next assessed the synaptic morphology by observation of GFP fluorescence in worms expressing both S129A-α-synuclein and SNB-1::GFP (GFP-tagged synaptobrevin); these worms were generated by microinjection of S129A-α-synuclein plasmid into the strain juls1, which expresses SNB-1::GFP in a subset of ventral nerve cord neurons. GFP fluorescence of SNB-1::GFP was broadly diminished or extremely weak in the middle of the nerve cord in S129A-Tg worms (Fig. 2E), whereas the SNB-1::GFP fluorescence was preserved in S129D-Tg worms as in juls1. Taken together, S129A-α-synuclein, a non-phosphorylatable form, caused severe dysfunction in neurons of worms, suggesting that phosphorylation of α-synuclein at Ser-129 protects against neurotoxicity of α-synuclein in C. elegans neurons.

S129A-α-Synuclein Showed High Affinity to Membranes—Next, we examined the biochemical properties of S129A or S129D versus WT α-synuclein expressed in C. elegans. Worm protein lysates were sequentially extracted by Tris-HCl buffer, then by Tris-HCl buffer containing Triton X-100, then by Sarkosyl, and finally by SDS (Fig. 3A). The amount of α-synuclein extracted in each fraction was assessed by immunoblotting. The most insoluble α-synuclein species, which were
FIGURE 2. Overexpression of S129A-α-synuclein in C. elegans caused severe phenotypic defects. A, representative pictures of synchronized cultures of N2, S129A-Tg, and S129D-Tg worms 55 h after hatching. The inset in the S129A-Tg panel shows the coiler phenotype. Scale bar = 500 μm. B, growth rate of each strain assessed by measuring the body length. The growth of S129A-Tg was significantly delayed. Data represent mean ± S.E., n = 20, *, p < 0.0001 when compared with N2. C, motor function of each strain at the age of day 1 (L2 larva), day 3 (young adult), and day 8 was assessed by thrashing assay. Each worm was soaked in M9 buffer, and the frequency of body bends was counted. Data represent mean ± S.E., n = 15, *, p < 0.0001 when compared with N2. D, overall appearances of representative synWT-Tg, S129A-Tg, and S129D-Tg worms (left, middle, and right, respectively). A majority of S129A-Tg worms exhibited an egg-laying defect. Bar = 500 μm. E, synaptic abnormalities in L2 stage worms expressing SNB-1::GFP as detected by the GFP fluorescence. S129A-α-synuclein was overexpressed in the strain jul1(Punc-25:snb-1::gfp). Arrowheads indicate the sites at which fluorescence is broadly diminished. Bar = 10 μm.
expected to be extracted in the SDS fraction, were hardly detected in both synWT-Tg and S129A-Tg worms. On the other hand, α-synuclein in the Triton X-100 fraction, in which membrane-bound proteins were highly concentrated, was significantly increased in S129A-Tg worms (Fig. 3B). We also examined Tg worms expressing A30P-α-synuclein (A30P-Tg), which is known to have lower membrane binding properties (16, 19, 20), and confirmed that the amount of A30P-α-synuclein in the Triton X-100 fraction was low (Fig. 3B). From these data, we hypothesized that the increased membrane binding of α-synuclein is related to the dysfunction caused by S129A-α-synuclein.

**Phosphorylation of α-Synuclein Protects against Neuronal Dysfunction along with Reduction in Its Membrane Binding Property**—To ensure the correlation among phosphorylation, membrane binding property, and neurotoxicity of α-synuclein, we next generated Tg worms overexpressing A30P-α-synuclein (A30P-Tg), which is expected to retain low membrane binding property with a lack of phosphorylation. We generated three independent A30P/S129A-Tg lines and found that all three lines showed nearly normal motor activities (Fig. 4A). In addition, the overall morphologies (Fig. 4B) as well as the growth rates of A30P/S129A-Tg worms (data not shown) were also normal. The levels of α-synuclein in A30P/S129A-Tg lines were confirmed to be comparable with those in other α-synuclein Tg lines (Fig. 4C). We then tested the membrane binding property of A30P/S129A-α-synuclein by detergent fractionation analysis and found that the amount of A30P/S129A-α-synuclein extracted in the Triton X-100 fraction was significantly lower than those in S129A-Tg and synWT-Tg worms (Fig. 4, D and E). We also found that the membrane binding property of S129D-α-synuclein was lower than that of WT-α-synuclein (Fig. 4, D and E). These results suggest that the dysfunction might have been caused by nonphosphorylatable α-synuclein exhibiting high membrane binding property and that phosphorylation at Ser-129 is sufficient to reduce α-synuclein-mediated impairment possibly by lowering its membrane binding property.

**S129A-α-Synuclein Exhibited Increased Targeting to Membranes in SH-SYSY Cells**—We next sought to obtain the visible evidence that phosphorylation regulates the membrane localization of α-synuclein. As C. elegans neuronal cell bodies are too small to examine the subcellular localization of expressed proteins by immunostaining, we used human neuroblastoma SH-SY5Y cells for examining the distribution pattern of WT, S129A, and S129D-α-synuclein. A prior study suggested that a membrane-bound fraction of α-synuclein can be visually seen by treatment with digitonin, which selectively permeabilizes the plasma membrane, releasing soluble and cytoplasmic α-synuclein (34). In the absence of digitonin treatment, all α-synuclein species similarly exhibited diffuse distribution patterns in the cytoplasm, masking a small fraction associated with membrane structures (Fig. 5A, upper panels). Phosphorylated α-synuclein at Ser-129 was also detected in cells expressing WT-α-synuclein but not in those expressing S129A or S129D-α-synuclein (Fig. 5A, lower panels). When cells were permeabilized with digitonin, residual α-synuclein associated with membranes was detected as a granular staining pattern (Fig. 5B). We found that the cells expressing WT and S129D-α-synuclein showed relatively moderate residual staining of α-synuclein, whereas those expressing S129A-α-synuclein showed much stronger staining (Fig. 5B). This increase of residual granular staining was quantitatively verified by the measurement of fluorescence intensity (Fig. 5C). We also confirmed that the expression levels of α-synuclein were comparable among the cells expressing these α-synuclein species (Fig. 5D). We also performed the same experiment using HEK293 cells and found that S129D-α-synuclein exhibited decreased localization to membranes when compared with WT and S129A-α-synuclein (supplemental Fig. 2), suggesting that mimicking phosphorylation-
tion reduces the membrane localization in non-neuronal cells. As the residual membrane structure after digitonin treatment is thought to be mainly composed of lipid rafts (34), we double-stained for flotillin, a known marker for lipid rafts, and we could confirm clear co-localization of \( \alpha \)-H9251-synuclein with flotillin after digitonin treatment (supplemental Fig. 2). Together, these results suggest that \( \alpha \)-H9251-synuclein phosphorylation reduces its localization to membranes that include lipid rafts.

Overexpression of S129A-\( \alpha \)-Synuclein in C. elegans Neurons Caused Up-regulation of Daf-16/FOXO Signaling Pathway—

To identify the downstream molecular pathway of the overexpression of nonphosphorylatable and membrane-associated \( \alpha \)-synuclein in C. elegans neurons, we performed genome-wide expression analysis of S129A-Tg worms and compared the results with those of N2, synWT-Tg, and A53T-Tg worms, using the Affymetrix C. elegans genome array. Gene expression profiles of \( \sim \)22,500 transcripts were compared among the strains, and the genes whose expression was significantly up-regulated or down-regulated more than 1.5-fold in S129A-Tg worms when compared with all other strains were selected. As a result of data mining and confirmation by real-time PCR analysis, we found that the expression levels of a set of representative DAF-16/FOXO target genes, i.e. sod-5, sod-3, mtl-1, and dod-3, were dramatically up-regulated in S129A-Tg worms (Fig. 6A). DAF-16/FOXO is a well known forkhead transcription factor responsible for various important physiological functions, e.g. lifespan extension and stress response. To gain insights into the significance of the up-regulation of DAF-16 targets, S129A-Tg worms were crossed with daf-16(mu86) mutant, which lacks functional DAF-16 protein expression but shows nearly normal development (35, 36). We found that the S129A-Tg;daf-16(mu86) worms were dramatically sick and almost nonviable, whereas the daf-16(mu86) mutant grew up without major defects (Fig. 6, B and C). These results suggest that the up-regulation of the daf-16/FOXO signaling pathway detected in S129A-Tg worms may represent a neuroprotective response against \( \alpha \)-synuclein-induced neuronal dysfunction.

DISCUSSION

In this study, we examined the role of Ser-129 phosphorylation of \( \alpha \)-synuclein in its neurotoxicity using a transgenic C. elegans model and suggested a possible molecular mechanism of phosphorylation-mediated neuroprotection against \( \alpha \)-synuclein-induced dysfunction. Although previous studies

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**FIGURE 4.** A30P/S129A- and S129D-\( \alpha \)-synuclein cause little dysfunction and have lower membrane binding property. A, liquid thrashing assay. Three independent A30P/S129A-Tg worms were normal, in contrast to severe motor dysfunction in two S129A-Tg lines. Data represent mean \( \pm \) S.E. \( n = 15 \). *, \( p < 0.0001 \). B, overall appearance of representative A30P/S129A-Tg worm, showing normal morphology. Bar = 500 \( \mu \)m. C, immunoblot analysis showing the expression levels of \( \alpha \)-synuclein (\( \alpha \)-Syn) in the indicated lines at the young adult stage. D, detergent fractionation analysis. The extracted proteins were analyzed by immunoblotting with an anti-\( \alpha \)-synuclein antibody LB509. Positive \( \alpha \)-synuclein immunoreactivities were detected only in the Tris-HCl and Triton X-100 fractions. E, the percentages of the membrane-bound \( \alpha \)-synuclein (Triton X-100 fraction) that composed the total \( \alpha \)-synuclein (sum of \( \alpha \)-synuclein in the Tris-HCl and Triton X-100 fractions) were quantified by the densitometric measurement of the bands. Data represent mean \( \pm \) S.E. from four independent tests. *, \( p < 0.05 \) when compared with synWT-Tg.
have reported the toxic effects of S129A or S129D-α-synuclein in neurons of transgenic rat or Drosophila models (25–28), detailed molecular mechanisms underlying the toxicity remained elusive. Here we demonstrated a strong correlation among α-synuclein binding to membrane, phosphorylation at Ser-129, and neuronal dysfunction. Although we cannot fully exclude the possibility that membrane binding of α-synuclein is independent of the dysfunction, our present data imply a mechanism whereby phosphorylation of α-synuclein reduces neuronal impairment through a reduction in the amount of membrane-bound α-synuclein. We also found that the inhibition of the Daf-16/FOXO signaling pathway dramatically exacerbated the neuronal dysfunction caused by S129A-α-synuclein.

In Tg rats that overexpress human α-synuclein by adeno-associated virus-mediated gene transfer, S129A-α-synuclein displayed higher neurotoxicity when compared with WT- and S129D-α-synuclein (25, 26), which was supplemented by an immunoelectron microscopic analysis showing a close localization of S129A-α-synuclein to cellular membranes (26). In addition, S129D-α-synuclein expressed in budding yeast exhibited decreased plasma membrane association (37). These observations are consistent with our results, suggesting the conserved

![Image of fluorescence and immunoblot analysis](image-url)
mechanism of regulation of neurotoxicity and membrane binding by Ser-129 phosphorylation.

Our finding that introduction of A30P mutation reduced the neuronal dysfunction caused by S129A/H9251-synuclein seems a little paradoxical, given the effect of A30P as a disease-causing mutation (2). Although the reason for this discrepancy remains unknown, A30P/H9251-synuclein may have a somewhat different nature of toxicity, which has been implied by previous studies.

**FIGURE 6.** Up-regulation of expression of Daf-16/FOXO signaling pathway genes caused by pan-neuronal overexpression of S129A-α-synuclein. A, real-time RT-PCR analysis of mRNA levels of DAF-16 targets sod-5, sod-3, mtl-1, and dod-3. Relative expression levels when compared with those in wild-type N2 are shown. Data represent mean ± S.E., n = 4, *p < 0.0001 when compared with N2 and synWT-Tg. B, stereoscopic views of S129A-Tg worms with or without daf-16(mu86) allele, which are the progenies of a single adult placed 9 days before. Many progenies of S129A-Tg grew up after 9 days, whereas those of S129A-Tg;daf-16(mu86) hardly grew up. Bar = 500 μm. C, the average number of adults of indicated strains that are the progenies of a single adult placed 5 days before. Data represent mean ± S.E., n = 8, *p < 0.0001.
reporting that A30P-α-synuclein Tg mice did not show overt phenotype or pathology (38, 39). One possible pathogenic mechanism of A30P-α-synuclein would be the formation of neurotoxic prefibrillar oligomers (40, 41), which may not be working in some animal models including our Tg worms.

A portion of α-synuclein molecules is thought to be directly associated with membrane lipids (15–17), and importantly, it has been shown that α-synuclein harbors a high affinity for negatively charged, acidic phospholipids, e.g. phosphatidylserine or phosphatidylinositol (15, 18, 19, 42). Thus, it is reasonable to assume that Ser-129 phosphorylation provides α-synuclein with negative charges, which in turn lowers the electrostatic interaction of α-synuclein with acidic phospholipids, leading to the dissociation of α-synuclein from cellular membranes. Indeed, a prior study demonstrated that phosphorylation at Ser-129 reduced the affinity of α-synuclein to membranes in vitro (24). However, we should be careful about this notion as another in vitro study failed to show the perturbing effect of phosphorylation on its membrane-bound conformation (43), and a biochemical approach using synaptosomal membranes showed a lack of modifying effect of phosphorylation on membrane binding of WT α-synuclein (44).

From the immunocytochemical point of view, however, our studies using human cell lines support the notion that Ser-129 phosphorylation renders α-synuclein to dissociate from membrane structures. As for the difference in the degree of membrane targeting of WT α-synuclein between SH-SY5Y and HEK293 cells, this might be explained by the difference between neuronal versus non-neuronal cells or by the difference in the amount of phosphorylated α-synuclein between these cells. It was hard to examine the latter possibility as the quantitative approach by immunoblotting failed to detect phosphorylated α-synuclein in both cell lines, although phosphorylation was cytochemically detected. We should also carefully argue about the effect of S129D mutation as another study showed a difference in the structural property between in vitro purified S129D mutant and α-synuclein phosphorylated by CK1 (43). However, the molecular dynamics of α-synuclein in vivo are expected to be different from the in vitro state, considering the situation such that α-synuclein physiologically occurs in oligomer formations such as tetramers (45).

The notion that the membrane-bound form of α-synuclein causes neuronal dysfunction or toxicity is supported by previous studies using animal models, in which overexpression of α-synuclein caused defects in vesicle trafficking (46). In yeast, the earliest defect by α-synuclein overexpression was the block of endoplasmic reticulum–Golgi trafficking, and the toxicity was rescued by overexpression of Rab1, Rab3A, or Rab8A, all of which are involved in different steps in vesicle trafficking (47, 48). In addition, a recent study using α-synuclein Tg C. elegans showed that α-synuclein toxicity was rescued by Vps41, a protein involved in lysosomal trafficking (49). We have also shown that Tg C. elegans pan-neuronally overexpressing α-synuclein displayed defects in synaptic vesicle endocytosis (29); in this worm, the amount of membrane-bound α-synuclein was dramatically increased by the inhibition of AP-2, an adaptor molecule involved in the early steps of clathrin-mediated endocy-
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We also revealed an involvement of the Daf-16/FOXO signaling pathway downstream of the overexpression of nonphosphorylatable α-synuclein. It is well known that Daf-16/FOXO pathway is activated by insulin signaling or cellular stresses and involved in a broad range of bioregulation from metabolism to longevity (35, 52, 53). Although little is known about the involvement of FOXO signaling in Parkinson disease, a recent study showed that LRRK2, another gene causative to the autosomal dominant form of familial Parkinson disease, causes neurotoxicity via up-regulation of FOXO1 pathway in Drosophila (54). Among a number of target genes of Daf-16 thus far identified, up-regulation of antioxidative stress genes, i.e. sod-5, sod-3, and mtl-1, was especially prominent in our Tg worms overexpressing S129A-α-synuclein, implicating oxidative stress in the neuronal defects by S129A-α-synuclein. This view is supported by our finding that the genetic deletion of daf-16 dramatically enhanced the defects in S129A-Tg worms, although it still remains possible that this phenotypic aggravation is rather caused by an epistatic effect of daf-16. It also remains possible that other Daf-16/FOXO target genes or pathways in addition to antioxidative stress genes may play critical roles in response to the dysfunction caused by S129A-α-synuclein. Thus, it would further be required to examine the changes in various FOXO target genes in worms as well as other models or human pathology of α-synucleinopathies.

Based on the data derived from this study, three strategies to reduce the neurotoxicity of α-synuclein would be considered: (i) inhibition of association of α-synuclein with cellular membranes, (ii) promotion of α-synuclein phosphorylation or inhibition of dephosphorylation of α-synuclein at Ser-129, and (iii) activation of FOXO signaling pathway. Gaining the ability to assess these strategies in other models involving higher-order organisms may pave the way to the establishment of novel therapeutic strategies for α-synucleinopathies.

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