# Effects of Serine 129 Phosphorylation on $\alpha$ -Synuclein Aggregation, Membrane Association, and Internalization<sup>\*</sup>

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Although trace levels of phosphorylated  $\alpha$ -synuclein ( $\alpha$ -syn) are detectable in normal brains, nearly all  $\alpha$ -syn accumulated within Lewy bodies in Parkinson disease brains is phosphorylated on serine 129 (Ser-129). The role of the phosphoserine residue and its effects on  $\alpha$ -syn structure, function, and intracellular accumulation are poorly understood. Here, co-expression of  $\alpha$ -syn and polo-like kinase 2 (PLK2), a kinase that targets Ser-129, was used to generate phosphorylated  $\alpha$ -syn for biophysical and biological characterization. Misfolding and fibril formation of phosphorylated  $\alpha$ -syn isoforms were detected earlier, although the fibrils remained phosphatase- and protease-sensitive. Membrane binding of  $\alpha$ -syn monomers was differentially affected by phosphorylation depending on the Parkinson disease-linked mutation. WT  $\alpha$ -syn binding to presynaptic membranes was not affected by phosphorylation, whereas A30P  $\alpha$ -syn binding was greatly increased, and A53T  $\alpha$ -syn was slightly lower, implicating distal effects of the carboxyl- on amino-terminal membrane binding. Endocytic vesicle-mediated internalization of pre-formed fibrils into non-neuronal cells and dopaminergic neurons matched the efficacy of  $\alpha$ -syn membrane binding. Finally, the disruption of internalized vesicle membranes was enhanced by the phosphorylated  $\alpha$ -syn isoforms, a potential means for misfolded extracellular or lumenal  $\alpha$ -syn to access cytosolic  $\alpha$ -syn. Our results suggest that the threshold for vesicle permeabilization is evident even at low levels of  $\alpha$ -syn internalization and are relevant to therapeutic strategies to reduce intercellular propagation of  $\alpha$ -syn misfolding.

 $\alpha$ -Synuclein ( $\alpha$ -syn)<sup>9</sup> is an abundant neuronal protein that reversibly binds to intracellular membranes. Unfolded cytosolic  $\alpha$ -syn is in dynamic equilibrium with the membrane-associated form that is partially constrained in an amphipathic  $\alpha$ -helical conformation (1-4). In healthy neurons,  $\alpha$ -syn presynaptic localization is mediated by direct binding to synaptic vesicles and is implicated in regulating vesicle trafficking and fusion machinery (5–7).  $\alpha$ -syn is also a key factor in the pathogenesis of Parkinson disease (PD) and other synucleinopathies, whereby it progressively adopts a  $\beta$ -sheet conformation prone to self-assembly. Several studies have reported that misfolded  $\alpha$ -syn multimers can be exchanged between adjacent neurons and further propagated by recruitment of unfolded  $\alpha$ -syn in recipient cells in a prion-like manner (8). The underlying pathways that govern this neuron-to-neuron movement are poorly understood, as is the basis for the conformational change.

 $\alpha$ -syn can be modified by multiple covalent post-translational modifications and truncations that may affect both folding and interneuronal transfer by limiting  $\alpha$ -syn flexibility, modifying membrane association, complex formation, and degradation (9). Almost 90% of  $\alpha$ -syn in Lewy bodies (LB) is phosphorylated on Ser-129 (Ser(P)-129), yet only 5% of  $\alpha$ -syn in normal brains is similarly modified (10–12). Whether Ser(P)-129  $\alpha$ -syn affects aggregation, secretion, or uptake is equivocal, and subtle effects that are undetectable at low levels of expression in healthy tissue could be manifest cumulatively during disease progression as a result of pathological enrichment.

In this study, we compared the biophysical and membrane/ cellular interactions of recombinant nonphosphorylated and Ser(P)-129  $\alpha$ -syn to address whether phosphorylation affects its pathogenic characteristics. Differentially phosphorylated isoforms were generated by co-expressing wild-type (WT) and PD mutant (A30P and A53T)  $\alpha$ -syn isoforms in *Escherichia coli* either without or with polo-like kinase 2 (PLK2), which uniquely phosphorylates Ser-129 on  $\alpha$ -syn (13–16). Surprisingly, phosphorylation increased the self-assembly of the purified  $\alpha$ -syn but had differential effects on membrane binding of monomeric  $\alpha$ -syn and the internalization of aggregated  $\alpha$ -syn isoforms. Although WT  $\alpha$ -syn membrane binding was not sig-



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<sup>&</sup>lt;sup>9</sup> The abbreviations used are: α-syn, α-synuclein; CIP, calf intestinal phosphatase; LB, Lewy bodies; PD, Parkinson disease; SN, substantia nigra; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

nificantly affected, phosphorylation greatly enhanced that of A30P  $\alpha$ -syn and reduced A53T  $\alpha$ -syn binding. Moreover, despite the change in fibril formation, we observed that the relative effects of Ser(P)-129 on  $\alpha$ -syn membrane binding were similar to its effects on  $\alpha$ -syn internalization, suggesting that membrane binding is a rate-limiting step in  $\alpha$ -syn internalization. However, endocytic vesicle rupture tended to be more pronounced with phosphorylated  $\alpha$ -syn, even with  $\alpha$ -syn isoforms with low levels of internalization, suggesting that even small amounts of  $\alpha$ -syn loading per vesicle are sufficient for membrane permeabilization. These results are relevant to therapeutic strategies to reduce interneuronal  $\alpha$ -syn transfer, and suggest that targeting  $\alpha$ -syn binding to extracellular membranes may be a promising approach.

#### **Experimental Procedures**

*Preparation and Purification of* α-*Synuclein Fibrils*—Recombinant α-synuclein was expressed in *E. coli* with or without PLK2, purified as described previously (17), and stored in 20 mM Tris-Cl, pH 7.4. Fibril fractions were prepared by incubating purified proteins (1.5 mg/ml) at 37 °C and shaking at 200 rpm for ~7–10 days. Samples for electron microscopy (EM) and circular dichroism (CD) were prepared at every 3–4-day intervals. The extent of Ser-129 phosphorylation was assessed by Western blotting with an anti-Ser(P)-129 antibody (11A5 mouse monoclonal generously supplied by Elan Pharmaceuticals). Nonphosphorylated and phosphorylated α-syn were also analyzed by reverse phase HPLC (0.1% trifluoroacetic acid; 60% acetonitrile) using a C18 (Phenomenex) column (3-μm particle; 300-Å pore) with protein detection at 254 nm.

*Circular Dichroism Spectroscopy*—Far-UV CD spectra of purified fibrils in protein buffer were collected at room temperature using a Jasco-J175 spectrophotometer and 0.1-cm cuvette. Data were acquired at a step size of 0.2 nm with an averaging time of 3 s.

Electron Microscopy and Image Processing—Purified  $\alpha$ -synuclein fibrils were adhered to glow-discharged, carbon-coated copper grids. Grids were stained, washed, and inspected as described previously (18).

Digestion with Proteinase K (PK)—An aliquot of 2  $\mu$ g/ml proteinase K (Sigma) was added to 1.5 mg/ml purified  $\alpha$ -synuclein fibrils. 15  $\mu$ l of a fibril solution was taken out prior to the addition of PK, as a control for the untreated sample, and at different time points of treatment, an aliquot of 15  $\mu$ l was taken for analysis. Each aliquot was quenched by the addition of SDS sample buffer and boiled for 10 min before being resolved on a 16% Tris-glycine SDS-PAGE for Western blotting.

α-Syn Membrane Binding—Synaptosomal membranes prepared from α-syn-deficient mice were incubated for 10 min at 37 °C with 1.5 mg/ml α-syn-deficient cytosol supplemented with 3 µg of recombinant non-phosphorylated or phosphorylated WT, A30P, or A53T α-syn as described (4). Membranes were then centrifuged at 24,000 × g for 10 min, washed twice with buffer to remove excess α-syn (25 mM HEPES, 125 mM KOAc, and 2.5 mM MgCl<sub>2</sub>), and resuspended in 1% SDS buffer for Western blotting. Bound HRP-conjugated anti-mouse or anti-rabbit IgG was revealed by chemiluminescence using ECL Plus (GE Healthcare) and quantified with a Li-Cor Odyssey Fc imager and Image Studio software (Li-Cor). All measurements were done in the linear range using standardized dilutions of mouse brain lysates or purified  $\alpha$ -syn. Percentage of ratios of densitometric values for anti-syn immunoreactive bands of bound to total (bound and unbound) were calculated in each experiment (n = 6) and assessed using one-way analysis of variance and Tukey's post hoc test.

*Cell Culture*—HeLa cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Wisent) supplemented with 10% fetal bovine serum (Sigma). For staining and immunochemical studies, cells were seeded at a density of 15,000 cells/cm<sup>2</sup> on 12-mm glass coverslips.

Mesencephalic cultures were prepared according to a previously described protocol, with minor variations (19). Briefly, dissociated neurons micro-dissected from the substantia nigra (SNc) or ventral tegmental area (VTA) of P0–P2 C57BL6 mice were seeded on a monolayer of cortical astrocytes grown on collagen/poly-L-lysine-coated glass coverslips at a density of 100,000 cells/ml. Treatments with human  $\alpha$ -syn fibrils, previously sonicated, were performed at 7 days *in vitro* for 24 h.

SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, and 10  $\mu$ g/ml ciprofloxacin. Cells were maintained in a 37 °C incubator with 5% CO<sub>2</sub>. SH-SY5YchGal3 cells were made according to the protocol as described previously (20).

*Fibril Treatment*— $\alpha$ -syn fibrils (5 µg/ml) were sonicated for 30 s and added to the cell cultures. In some experiments, cultures were pre-treated with 80 µM dynasore for 30 min at 37 °C, which inhibits the endocytosis of clathrin-coated vesicles. Cells were subsequently co-incubated with 80 µM dynasore and/or 5 µg/ml fibrils for 20 min, after which the cultures were prepared for immunocytochemistry.

Confocal Imaging-Cultures were fixed for 30 min with 4% paraformaldehyde in PBS and permeabilized, and nonspecific binding sites were blocked. Cells were incubated overnight with a primary antibody solution containing 1% bovine serum albumin (BSA), 0.1% Triton X-100 in PBS, 5% goat serum, and 0.02% NaN<sub>3</sub>. HeLa cells were stained for 1 h with 100 nm actin stain 488 fluorescent phalloidin in PBS and washed three times in PBS before incubation for 1 h with the appropriate Alexalabeled secondary antibodies (Invitrogen). Primary antibodies used were rabbit tyrosine hydroxylase (TH) (Millipore, 1:2000) and  $\alpha$ -syn (Invitrogen, 1:1000). After the final washes, coverslips with HeLa cells were placed on slides with mounting media (Vectashield, Vector Laboratories) containing 4,6-diamidino-2-phenylindole (DAPI) to stain double-stranded DNA. Digital images (4-5 per condition in each experiment) were captured through ×40 objectives using a Leica 6000 microscope or LSM 750 confocal and Volocity image analysis system or Zen software.

For neuronal cultures, images were acquired using an Olympus Fluoview FV1000 confocal microscope (Olympus) using a  $\times 60$  oil-immersion objective. Ten TH-positive dopamine neurons were selected from each coverslip. All image quantification was performed using ImageJ (National Institutes of Health) software. Background correction was first applied at





the same level for every image analyzed. TH images were then binarized, and the mask of these images was applied on the  $\alpha$ -syn images. Only the  $\alpha$ -syn signal co-localized with the TH signal was quantified. The total area of the  $\alpha$ -syn signal was normalized on the total area of the TH signal. All quantification was blinded, and at least eight coverslips for each condition, from two different cultures, were used.

Vesicle Rupture Assays-All vesicle rupture assays with the purified recombinant  $\alpha$ -syn were done in a blinded fashion. First, protein and solute concentrations, as well as total volume, were standardized across all four samples, according to previously published aggregation conditions (20). Specifically, 200  $\mu$ l of purified  $\alpha$ -syn (1 mg/ml) was prepared in a pH 7.4 buffer containing the following solute concentrations: 323.3 mM NaCl, 20 mm Tris-HCl, 9 mm Na<sub>2</sub>HPO<sub>4</sub>, 2.43 mm KCl, and 1.62  $m_M KH_2PO_4$ . Sample preparations were then incubated for 3 days at 37 °C under constant agitation, followed by storage at 4 °C. Aggregates were fluorescently labeled with DyLight 488 *N*-hydroxysuccinimide ester fluorophores (Thermo Scientific), according to the manufacturer's protocol prior to use. Briefly, 100  $\mu$ l of aggregated protein at 1 mg/ml was dialyzed into 0.1 M sodium phosphate buffer, pH 8.0, for 2 h using 10,000 molecular weight cutoff minidialysis units (Thermo Scientific). The protein solution was then transferred to the vial containing the dye and was labeled as per the supplier's instructions for 30 min at room temperature. After incubation, the labeling reaction was quenched with 40 mM Tris (final concentration), and extensive dialysis was performed using 1,000 molecular weight cutoff dialysis units into a buffer containing 40 mM Tris and 150 mM NaCl for about 24 h to remove excess unlabeled dye.

SH-SY5Y mCherry-galectin 3 (chGal3) cells were allowed to adhere to fibronectin (Sigma)-treated glass coverslips and fixed with 3.7% formaldehyde (Polysciences) in 0.1 M PIPES buffer, pH 6.8, for 5 min. DAPI stain was then performed for 20 min in PBS. Coverslips were then mounted on glass slides and allowed to dry before image acquisition. Images were collected with a DeltaVision microscope (Applied Precision) equipped with a digital camera (CoolSNAP HQ; photometrics), using either a 1.4-numerical aperture  $\times$ 100 objective lens or a 1.42-numerical aperture  $\times$ 60 objective lens. Images were then deconvolved with SoftWoRx deconvolution software (Applied Precision). Tiff images and quantification data were collected from each image data file using Imaris software (Bitplane).

Deconvolved images were analyzed for puncta formation by use of the Surpass Mode of the Imaris software package (Bitplane). Specifically, a three-dimensional surface was created around chGal3+ puncta by designing an algorithm for each experiment that specifically detected punctate events that increased in intensity sufficiently above background fluorescence. This same algorithm was uniformly applied to each image in the data set, and the number of surfaces created by the algorithm was divided by the number of cells in the field to measure puncta per cell. Each experiment collected at least 20 images per treatment type.

Algorithm-detected values for number of chGal3+ puncta per cell were pooled across all experiments for statistical analysis. Values for mean number of chGal3+ puncta per cell induced by each  $\alpha$ -syn treatment type from at least three inde-



FIGURE 1. Generation and purification of Ser-129-phosphorylated  $\alpha$ -syn. *A*, Western blot of phosphorylated  $\alpha$ -syn and total  $\alpha$ -syn purified from *E. coli* expressing WT, A30P, or A53T  $\alpha$ -syn in the absence or presence of PLK2. *Lower* bands show a Coomassie stain of the purified protein. *B*, HPLC elution of recombinant  $\alpha$ -syn generated in the absence or presence of PLK2. *C*, Western blots probed with anti-Ser(P)-129  $\alpha$ -syn antibody (*left blot*) and total  $\alpha$ -syn (*right blot*) showing dephosphorylation of A53T  $\alpha$ -syn by CIP (alkaline phosphatase).

pendent experiments were compared with one another and to untreated cells by use of one-way analysis of variance and Tukey's post hoc multiple comparison test. When a mean number of chGal3+ puncta per cell values from each experiment are normalized for Ser(P)-129  $\alpha$ -syn isoforms relative to their non-Ser(P)-129 counterparts in the same experiment (set to 1), unpaired *t* tests were performed to compare each  $\alpha$ -syn Ser(P)-129 isoform to its non-Ser(P)-129 counterpart.

#### Results

Generation and Purification of Ser(P)-129  $\alpha$ -syn—PLK2, which phosphorylates  $\alpha$ -syn solely on serine 129 (13–16), was co-expressed with  $\alpha$ -syn in *E. coli* to generate highly phosphorylated recombinant  $\alpha$ -syn. As shown in Fig. 1*A*, only  $\alpha$ -syn expressed in the presence of PLK2 was labeled by a Ser(P)-129specific antibody (mouse monoclonal 11A5). To evaluate whether all or some of the  $\alpha$ -syn was post-translationally modified, we measured the relative elution profiles following HPLC separation of nonphospho- and phosphoprotein. HPLC elution not only revealed distinct profiles of the phosphorylated and non-phosphorylated  $\alpha$ -syn eluted but also that nonphosphorylated  $\alpha$ -syn was undetectable when PLK2 was co-expressed (Fig. 1*B*). Interestingly, phosphorylation shifted the HPLC elution of each  $\alpha$ -syn, but both mutants were affected more than the WT  $\alpha$ -syn. Treatment of phospho- $\alpha$ -syn with calf intestinal



FIGURE 2. Effects of Ser-129 phosphorylation on  $\alpha$ -syn membrane binding. Synaptic membranes from  $\alpha$ -syn-deficient mice were incubated with 1.5 mg/ml  $\alpha$ -syn-deficient brain cytosol and 3  $\mu$ g of recombinant human  $\alpha$ -syn, either non-phosphorylated (–) or phosphorylated (*p*). The *top panel* shows representative Western blots of membrane-bound and unbound  $\alpha$ -syn, and the *lower panel* shows  $\alpha$ -syn binding as a percent of total  $\alpha$ -syn (mean  $\pm$  S.E., n = 6, \*, p < 0.05).

phosphatase (CIP; alkaline phosphatase) removed the Ser(P)-129 epitope  $\alpha$ -syn but had no effect on the total  $\alpha$ -syn (Fig. 1*C*).

Membrane Binding Properties of Monomeric Ser(P)-129  $\alpha$ -syn—We previously characterized the membrane binding of purified WT and PD-linked mutant  $\alpha$ -syn to presynaptic membranes isolated from  $\alpha$ -syn-deficient mouse brain (4, 16). However, the impact of  $\alpha$ -syn phosphorylation at its carboxyl-terminal domain, the primary pathological post-translational modification, though distal to the amino-terminal membrane binding domain, is unknown. Therefore, we assessed normal and mutant  $\alpha$ -syn membrane binding with or without Ser(P)-129. Synaptosome membranes were incubated for 10 min at 37 °C with either nonphosphorylated or phosphorylated  $\alpha$ -syn, washed, and probed for bound  $\alpha$ -syn. Phosphorylation of WT  $\alpha$ -syn had no detectable effect on its binding, whereas A30P and A53T  $\alpha$ -syn binding was differentially affected (Fig. 2). Strikingly, although A30P  $\alpha$ -syn bound poorly to membranes as reported by other studies (21–24), the A30P  $\alpha$ -syn phosphoprotein showed a significant enhancement in membrane binding capacity, comparable with WT levels. In contrast, phosphorylation of Ser-129 reduced the A53T  $\alpha$ -syn recovery in the membrane fraction. Thus, phosphorylation affected the membrane binding of each  $\alpha$ -syn isoform differently.

Analysis of in Vitro Aggregation—A primary question arising from the observation that the detergent-insoluble and aggregated  $\alpha$ -syn in LB is highly phosphorylated is whether the phosphorylation influences  $\alpha$ -syn self-assembly. To address the role of Ser(P)-129 in  $\alpha$ -syn fibril formation, the three  $\alpha$ -syn isoforms and their phosphorylated versions were incubated in neutral pH buffer at 37 °C with constant shaking, and aliquots were periodically removed to assess circular dichroism (CD) spectra and for analysis by electron microscopy (EM). As shown by the CD spectra in Fig. 3*A*, the Ser-129-phosphorylated  $\alpha$ -syn isoforms appeared to transition earlier from random coil to typical  $\beta$ -sheet (minima at ~220 nm). In accord, all  $\alpha$ -syn versions bearing Ser(P)-129 were also prone to form longer and more intricate fibril networks before their nonphosphorylated counterparts as shown by negative-stain electron microscopy (Fig. 3*B*).

Pathological  $\alpha$ -syn fibrils isolated from PD or dementia with Lewy bodies brains display pronounced resistance to proteinases (25–27). However, the fibrils generated from phosphorylated  $\alpha$ -syn did not show enhanced tolerance to proteinase K compared with nonphosphorylated forms (A30P is shown in Fig. 3*C*). Although the majority of the  $\alpha$ -syn fibrils were not SDS-resistant and with similar mobility as monomeric  $\alpha$ -syn, multiple high molecular weight  $\alpha$ -syn species can be detected after 10 days of incubation. More low mobility  $\alpha$ -syn bands were present in the phosphorylated  $\alpha$ -syn lanes, but there was no obvious difference in proteinase K sensitivity between the phosphorylatedandnon-phosphorylatedfibrils.Similartomonomeric Ser(P)-129  $\alpha$ -syn, these phosphorylated A30P fibrils were also sensitive to dephosphorylation by CIP (Fig. 3*D*).

Cellular Uptake of Ser(P)-129  $\alpha$ -syn by Non-neuronal and Neuronal Cells-To evaluate the effects of Ser-129 phosphorylation on  $\alpha$ -syn cellular uptake, HeLa cells were exposed to sonicated preformed  $\alpha$ -syn fibrils for 20 min, and uptake was evaluated by immunofluorescence. These non-neuronal cells have a large cytoplasmic area that is more amenable to internalization analyses than neurons. In all experiments, antibodies to  $\alpha$ -syn labeled puncta of various sizes, suggestive of  $\alpha$ -syn accumulation either at the cell surface or following internalization. These were compared with control experiments with either no added extracellular aggregates or with preformed fibrils composed of islet amyloid polypeptide (Fig. 4). Neither of these control conditions induced  $\alpha$ -syn immunoreactivity, indicating that  $\alpha$ -syn labeling detected after the exposure to extracellular preformed  $\alpha$ -syn fibrils was indeed due to cell surface binding and internalization of the exogenous  $\alpha$ -syn. All fibrillar  $\alpha$ -syn isoforms showed some accumulation by HeLa cells, although Ser-129 phosphorylation affected internalization of WT and mutant  $\alpha$ -syn differentially. As with the binding to presynaptic membranes, internalization of WT and Ser(P)-129 WT  $\alpha$ -syn was not significantly different, but both PD mutants were significantly affected by phosphorylation. Similar to their respective membrane binding properties, Ser-129 phosphorylation increased A30P uptake but reduced that of A53T  $\alpha$ -syn.

To determine whether  $\alpha$ -syn uptake is mediated by dynamindependent endocytosis, we also performed experiments in the presence of a dynamin inhibitor (28). Blockade of endocytosis with dynasore eliminated the perinuclear  $\alpha$ -syn immunoreactivity such that the accumulated  $\alpha$ -syn was predominantly distributed along the cell periphery in close apposition to the plasma membrane (Fig. 4*A*). Moreover, quantification of the immunofluorescence indicated that the net recovery of  $\alpha$ -syn within the HeLa cells was largely unaffected (Fig. 4*B*). This pattern is consistent with the inhibition of endocytic retrieval and





FIGURE 3. **Characterization of**  $\alpha$ -**syn fibrils.** Purified non-phosphorylated (*WT*, *A30P*, *A53T*) or phosphorylated (*WT-p*, *A30P-p*, *A53T*-p)  $\alpha$ -syn were subjected to 200 rpm shaking at 37 °C. At the indicated times, aliquots of  $\alpha$ -syn were assessed for protein secondary structure as determined by circular dichroism (*A*) and by negative stain transmission electron microscopy (*B*). Both parameters indicate that each phosphorylated  $\alpha$ -syn isoform displayed accelerated  $\beta$ -sheet formation and a higher propensity to form fibrils as compared with its non-phosphorylated version. *d*, day. Exposure to proteinase K (*C*) or calf intestinal phosphatase (*D*) indicated that proteinase sensitivity was unchanged and that the  $\alpha$ -syn fibril formation did not impede dephosphorylation, at least by 10 days of aging *in vitro* (A30P shown). *Scale bars* in *B* are 100 nm.

suggests that events preceding endocytosis, such as  $\alpha$ -syn membrane binding and sequestration, remain intact and that its internalization is vesicle-dependent.

We evaluated the accumulation of exogenous  $\alpha$ -syn fibrils by primary dopaminergic mouse neurons (19, 29), a cell popula-

tion known to be particularly vulnerable in PD. Isolated neurons from either substantia nigra (SN) or VTA, grown on coverslips for 7 days on an astrocyte monolayer, were treated with  $\alpha$ -syn fibrils and then examined for  $\alpha$ -syn uptake by confocal imaging after identification of neurons with TH immunoreac-





FIGURE 4. **Inhibition of dynamin-dependent endocytosis reduces**  $\alpha$ -**syn internalization.** *A*, HeLa cells were incubated with 5  $\mu$ g/ml of either naive untreated or islet amyloid polypeptide controls, non-phosphorylated (*WT*, *A30P*, *A53T*), or phosphorylated (*WT-p*, *A30P-p*, *A53T-p*)  $\alpha$ -syn fibrils. The sonicated fibrils were added in the presence or absence of the dynamin inhibitor, dynasore (240 nm). Figures are overlays of actin (phalloidin, *green*),  $\alpha$ -syn (*red*), and DAPI (nuclei, *blue*), with the middle *z*-stack chosen as illustration. *B*, quantification of  $\alpha$ -syn intensity between conditions in the presence and absence of dynasore (mean  $\pm$  S.E., n = 4-15, \*, p < 0.05).

tivity. To assess uptake specifically into dopaminergic neurons,  $\alpha$ -syn fluorescence that co-localized with tyrosine hydroxylase was quantified. After 24 h of exposure to sonicated pre-formed fibrils, punctate  $\alpha$ -syn labeling could be detected in TH-positive neurons treated with either phosphorylated or non-phosphorylated  $\alpha$ -syn (Fig. 5*A*). Quantification of the  $\alpha$ -syn fluorescence relative to TH revealed a pattern similar to uptake in the non-neuronal cells (Fig. 5*B*). For the SN dopaminergic neurons, there was a significant increase in Ser(P)-129-induced uptake of WT and A30P  $\alpha$ -syn and a minor trend to lower A53T  $\alpha$ -syn that was not statistically significant. In contrast, the impact of phosphorylation on fibril uptake into VTA dopaminergic neurons was closer to the HeLa cells; WT  $\alpha$ -syn was unaffected, whereas A30P uptake was increased, and A53T uptake was reduced.

Effects of  $\alpha$ -syn Fibrils on Vesicle Rupture—Prion-like propagation of abnormal  $\alpha$ -syn structure necessitates direct interaction between extracellular misfolded  $\alpha$ -syn and normal intracellular  $\alpha$ -syn in order for templating to occur. Thus, pathways that mediate  $\alpha$ -syn intermixing of intra- and extracellular  $\alpha$ -syn are of particular interest. To measure the ability of  $\alpha$ -syn to access the cytoplasm of cells following endocytosis, we exploited the relocalization of galectin-3 that occurs following vesicle permeabilization. The loss of vesicle membrane integrity permits galectin-3 access to lumenally oriented  $\beta$ -galactosides that are bound by the carbohydrate recognition domain present in galectin-3, and therefore it has been utilized to monitor vesicle rupture induced by bacterial and viral pathogens during infection (30, 31). We have previously reported that  $\alpha$ -syn aggregates induce galectin-3 relocalization to intracellular vesicles containing  $\alpha$ -syn following endocytosis (20). We therefore used this assay to investigate the ability of endocytosed non-phosphorylated and phosphorylated  $\alpha$ -syn to rupture vesicular membranes and to test the hypothesis that diseaseassociated familial missense mutations or post-translational modifications can differentially affect the potency of  $\alpha$ -syn entry via this disruptive mechanism.

We treated SH-SY5Y neuroblastoma cells stably expressing mCherry-galectin-3 (chGal3) with DyLight 488-labeled WT, A30P, or A53T  $\alpha$ -syn fibrils in either their non-phosphorylated or Ser-129-phosphorylated forms. Following a 24-h incubation in the cell culture medium, we assessed the formation of chGal3-positive puncta as a measurement of vesicle rupture induction. Each type of  $\alpha$ -syn was able to induce galectin-3 relocalization to intracellular punctate structures when compared with the diffuse cytoplasmic localization in untreated cells (Fig. 6A), and the profound co-localization of  $\alpha$ -syn and chGal3+ puncta implicates  $\alpha$ -syn as the causative agent of membrane permeabilization. Moreover, consistent with  $\alpha$ syn's ability to associate with vesicular membranes, Dylight488labeled  $\alpha$ -syn aggregates can frequently be seen adopting a curved or arc-shaped localization at the periphery of chGal3+ruptured vesicles.

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FIGURE 5. **Uptake of sonicated**  $\alpha$ -syn fibrils by primary SN and VTA DA neurons. *A*, representative confocal images showing a merge of TH immunoreactivity (*red*) and human  $\alpha$ -syn immunoreactivity after a 24-h incubation with human  $\alpha$ -syn WT, A30P, or A53T in a non-phosphorylated (*Non-phospho*) or phosphorylated (*Phospho*) form. *Scale bar*, 50  $\mu$ m. *B*, *bar graphs* show the percentage of  $\alpha$ -syn signal that co-localized with TH signal, normalized on TH area. Data for SN and VTA DA neurons are shown separately. Values represent the mean  $\pm$  S.E. of at least 80 neurons from two different cultures and statistically analyzed using a *t* test. \*, *p* < 0.00001.

To quantify vesicle rupture in a semi-automated unbiased fashion and to compare the relative abilities of each  $\alpha$ -syn species to cause this membrane permeabilization, we used algorithm-assisted identification of chGal3 puncta in untreated and treated cells. This algorithm reliably identified cytoplasmic chGal3 puncta, and notably all but one type of aggregated  $\alpha$ -syn, A30P, was able to induce a significant increase in the mean number of chGal3+ puncta per cell compared with untreated cells (Fig. 6B). Additionally, although Ser(P)-129 A30P was the only Ser(P)-129  $\alpha$ -syn treatment to induce a statistically significant increase in chGal3+ puncta per cell compared with its non-Ser(P)-129 counterpart, both Ser(P)-129 WT and Ser(P)-129 A53T showed trends above their nonphosphorylated counterparts, although not reaching statistical significance. Furthermore, when the mean number of chGal3+ puncta per cell values was normalized so that Ser(P)-129  $\alpha$ -syn isoforms were expressed as a fold change relative to their non-Ser(P)-129 counterparts within the same experiments, all Ser(P)-129  $\alpha$ -syn types show an  $\sim$ 50% increase in the mean number of chGal3+ puncta per cell relative to their non-Ser(P)-129 counterparts (Fig. 6C). Again, although the only mutant that exhibited a statistically significant increase was Ser(P)-129 A30P, both Ser(P)-129 WT and Ser(P)-129 A53T showed trends that were not statistically significant. This suggests that Ser-129 phosphorylation may impact the potency of vesicle rupture following endocytosis, as exemplified by the A30P  $\alpha$ -syn, which showed the greatest differential between its phosphorylated and non-phosphorylated forms in terms of membrane binding and internalization.

#### Discussion

 $\alpha$ -Syn undergoes multiple post-translational modifications (phosphorylation, ubiquitination, sumoylation, acetylation, nitration, and truncation). Of these, however, Ser-129-targeted phosphorylation is preferentially and consistently up-regulated in PD and related synucleinopathies (10, 12, 32–36). More than 90% of  $\alpha$ -syn is phosphorylated on Ser-129 in LB as compared with less than 5% in unaffected brains, suggesting that phosphorylation may influence its rate of aggregation and toxicity or its overall stability. However, the various studies that have examined the specific consequences of Ser-129 phosphorylation have failed to reach a consensus. In vitro biochemical phosphorylation assays have yielded inconsistent effects on  $\alpha$ -syn aggregation, with studies showing both increased and decreased  $\alpha$ -syn fibril formation (10, 37, 38). Similarly, modulation of Ser(P)-129  $\alpha$ -syn levels in cells by ectopic expression of various serine kinases known to phosphorylate  $\alpha$ -syn have also been equivocal on the function of phosphorylation, suggesting promotion (10, 39-42) or either inhibition or no effect (37, 43–48) on inclusion formation. Finally, the use of  $\alpha$ -syn S129A or S129D mutants to either prevent or mimic phosphorylation in various in vivo models has also yielded conflicting results. Co-expression of S129A  $\alpha$ -syn in flies rescued the neuronal loss induced by WT  $\alpha$ -syn expression, and correspondingly, S129D  $\alpha$ -syn was associated with increased pathology (39, 44, 47, 49, 50). However, contrary relationships were observed in other models, including yeast, mammalian cells, Caenorhabditis elegans, and rodents (51-53), and no phenotype was detected in  $\alpha$ -syn-deficient mice expressing either S129A or S129D transgenes (54). Not only has the function of Ser(P)-129  $\alpha$ -syn modification eluded explanation, but the stage at which this modi-



Effects of  $\alpha$ -Syn Phosphorylation

FIGURE 6. **Effects of Ser(P)-129**  $\alpha$ -syn fibrils on vesicle rupture assay. *A*, exposure of SH-SY5Y cells, stably expressing chGal3, to non-phosphorylated (*WT*, *A30P*, *A53T*) or phosphorylated (*WT-p*, *A30P-p*, *A53T-p*)  $\alpha$ -syn fibrils induced galectin-3 (*red*) and  $\alpha$ -syn (*green*) puncta. Regions of interest (*white square*) in the *left-most panels* are shown at higher magnification in the *right panels*. *White arrows* show co-localized galectin-3 (*red*) and  $\alpha$ -syn (*green*) puncta. Histograms show quantification of either chGal3+ puncta per cell (mean  $\pm$  S.E.,  $n \ge 3$ ; \*, p < 0.05 versus untreated; #, p < 0.05 versus non-phosphorylated  $\alpha$ -syn) (*B*) or impact of phosphorylation on vesicle rupture normalized to the non-phosphorylated isoforms (mean  $\pm$  S.E.,  $n \ge 3$ ; \*, p < 0.05 versus non-phosphorylated  $\alpha$ -syn) (*B*).

fication occurs is also unclear. There is some evidence that suggests that  $\alpha$ -syn can also be phosphorylated post-fibrillization (14, 15, 45, 55), perhaps as an effort to clear aggregated  $\alpha$ -syn (56), which is consistent with Ser(P)-129  $\alpha$ -syn accumulation during proteasome inhibition and elevated autophagymediated  $\alpha$ -syn degradation with PLK2 overexpression (16,

57). Because of the overall lack of consensus across these many studies, further confounded by variations in the extent of  $\alpha$ -syn phosphorylation and potential off-target effects of overex-pressed kinases and  $\alpha$ -syn mutants, we generated highly pure phosphorylated recombinant  $\alpha$ -syn to examine its biophysical and biological properties. This was done to avoid partial phos-



phorylation, noted by previous studies (55), which assessed mixtures of non-phosphorylated and phosphorylated  $\alpha$ -syn.

Our co-expression of PLK2 and  $\alpha$ -syn in *E. coli* provided consistent and ample yields of highly phosphorylated  $\alpha$ -syn. We noted that each of the Ser-129 phosphorylated  $\alpha$ -syn isoforms tended to lose random coil structure earlier, with a concomitant shift to  $\beta$ -sheet structure, in comparison with their non-phosphorylated counterparts. This structural change was also detected by EM as an earlier appearance of assembled fibrils by all three phosphorylated  $\alpha$ -syn isoforms. Thus, in our hands, this pro-aggregation effect is similar to experiments with  $\alpha$ -syn bearing carboxyl-terminal truncations (58–61). The phosphorylated fibrils remained sensitive to both phosphatase-mediated dephosphorylation and to proteinase K digestion. The latter properties suggest that the tertiary and quaternary structures of the assembled fibrils, although visible as long fibrils, were early stage fibrils and not compacted sufficiently to prevent enzymatic access. Also, given that the conversion from monomer to oligomer and fibril is continuous, all three forms are likely present in our preparations. Several lines of evidence suggest that  $\alpha$ -syn fibrils undergo some form of aging during seeding, not only in in vitro self-assembly assays (62), but also in  $\alpha$ -syn prion-like infection studies (63). Although the impact of phosphorylation on repeated seeding and propagation remains unclear, the recruitment of non-phosphorylated  $\alpha$ -syn by the phosphoprotein is particularly relevant to the spread of phosphorylated  $\alpha$ -syn from LB to proximal healthy neurons. Further study using the highly phosphorylated fibrils described here can help clarify the underlying mechanisms.

The involvement of post-translational modifications, particularly phosphorylation, on intercellular  $\alpha$ -syn movement linking prion-like mechanisms to the initiation and propagation of  $\alpha$ -syn pathology in PD is not understood. In this scheme, the internalization of extracellular misfolded  $\alpha$ -syn can be considered a multistep pathway involving cell surface interaction, internalization, and access to intracellular compartments containing endogenous  $\alpha$ -syn or turnover mediated by protein degradation machinery. Our previous report found only a small impact of  $\alpha$ -syn phosphorylation on its intracellular partitioning between membrane and cytosolic fractions (16), although others have reported an increase in nuclear localization (53, 64, 65) or reduced lipid binding (10, 32, 66). In contrast, this study used purified Ser(P)-129  $\alpha$ -syn and enabled us to measure its membrane binding properties and internalization more directly. Unlike the increased propensity to aggregate, Ser-129 phosphorylation did not uniformly impact membrane binding of monomeric WT, A30P, and A53T  $\alpha$ -syn with differential effects on the  $\alpha$ -syn isoforms. Phosphorylation of WT  $\alpha$ -syn had no effect on its membrane association, but it greatly enhanced A30P  $\alpha$ -syn binding and tended to reduce that of A53T  $\alpha$ -syn. Despite our lack of functional understanding of carboxyl-terminal modifications, these observations are consistent with evidence that long range carboxyl-terminal effects impact helix formation and binding efficiency of the aminoterminal amphipathic domain (67, 68). In addition, extracellular  $\alpha$ -syn binding to biological membranes may also involve coordination with lipids and additional protein components in

lipid raft domains via the carboxyl terminus (24, 69, 70). For example, there is evidence that the portion of the carboxyl-terminal domain containing the serine 129 residue of synaptic vesicle-bound  $\alpha$ -syn can interact with synaptobrevin (6). Although no protein interactors for the extracellular  $\alpha$ -syn carboxyl-terminal domain have been reported, recently heparan sulfate proteoglycans have suggested to act as receptors for  $\alpha$ -syn internalization (71).

The effect of phosphorylation on internalization of preformed  $\alpha$ -syn fibrils was assessed by immunofluorescence in both neuronal and non-neuronal cells, and it appeared to mirror the membrane binding properties of each  $\alpha$ -syn isoform. In both HeLa cells and dopaminergic neurons, the Ser-129 phosphorylation had only a subtle impact on WT  $\alpha$ -syn internalization, although the increase in SN neurons reached statistical significance. In contrast, phosphorylation had opposing effects on the  $\alpha$ -syn PD mutants, substantially increasing A30P and reducing A53T  $\alpha$ -syn accumulation. In the presence of the dynamin inhibitor dynasore, instead of the expected perinuclear accumulation, the  $\alpha$ -syn immunoreactivity was excluded from the cell interior and retained at the periphery adjacent to the plasma membrane. Despite the qualitatively different intracellular localization, the overall signal for each  $\alpha$ -syn isoform was not greatly reduced, indicating normal cell surface binding and sequestration, and consistent with a blockade of dynamindependent endocytosis (69, 72–74). Interestingly, the dynasore treatment accentuated the differential accumulation of the non-phosphorylated and phosphorylated WT  $\alpha$ -syn, raising the possibility that the endocytosed  $\alpha$ -syn may normally undergo trafficking to organelles with protein degradation machinery.

There is compelling evidence that extracellular misfolded higher order  $\alpha$ -syn multimers can undergo uptake by nearby cells, and a prion-like mechanism has been proposed for the spread of misfolded  $\alpha$ -syn by cell-to-cell transfer and self-perpetuating recruitment of normal  $\alpha$ -syn molecules into aberrant conformations (75-80). The cellular mechanisms by which extracellular or lumenal  $\alpha$ -syn can encounter endogenous  $\alpha$ -syn, which is predominantly cytosolic, are unclear, although one pathway that could mediate this encounter is via the rupture of endocytic vesicle membranes by internalized  $\alpha$ -syn (20). Damage to transport vesicles could therefore permit misfolded lumenal  $\alpha$ -syn to gain access to the cytosolic compartment where it may have an opportunity to propagate  $\beta$ -sheet structure to endogenous normal  $\alpha$ -syn. We therefore compared the relative abilities of WT, A30P, and A53T  $\alpha$ -syn fibrils to induce vesicle rupture in both their Ser-129 phosphorylated and nonphosphorylated forms. As measured by relocalization of mCherry-galectin-3, the effect of Ser-129 phosphorylation tended to increase vesicle rupture potency by about 50% for all  $\alpha$ -syn types above non-phosphorylated counterparts, although this was statistically significant only for A30P  $\alpha$ -syn. It should be noted that this assay measures vesicle permeability rather than  $\alpha$ -syn loading, such that any vesicle membrane damage would yield an all or none result. Thus, the inability of the nonphosphorylated A30P  $\alpha$ -syn to increase chGal3+ puncta above untreated controls may be governed by its poor association

with lipid membranes as compared with the other  $\alpha$ -syn isoforms.

Overall, our results show that, despite consistent effects of Ser-129 phosphorylation on fibril formation of each of the three  $\alpha$ -syn isoforms, there was considerable disparity with their membrane binding and internalization properties, as compared with vesicle rupture. From this, we propose that aggregation, per se, is likely not a sufficient determinant for the internalization of misfolded proteins, which is more strongly correlated with cell surface binding. This was most evident for the A30P  $\alpha$ -syn, which showed a significant enhancement of membrane binding in its phosphorylated state relative to the other  $\alpha$ -syn isoforms, and corresponded well with its internalization and vesicle rupture. Vesicle rupture by WT and A53T forms, however, did not show the same trends observed in membrane binding and internalization assay. In these cases, phosphorylation induced a slight increase in vesicle rupture that was not statistically significant. The simplest explanation for this discordance is that vesicle rupture is mediated by the oligomeric/ fibrillar forms of  $\alpha$ -syn, which were increased by phosphorylation in these studies. It is also tempting to speculate that these aggregation properties of phosphorylated  $\alpha$ -syn may underlie the early onset PD caused by  $\alpha$ -syn gene multiplication and the mutant forms. We suspect that the statistically significant increase in vesicle rupture observed by phosphorylated A30P, relative to its non-phosphorylated counterpart, is likely reflective of both Ser(P)-129 rescue of normally weak membrane binding/internalization in the non-Ser(P)-129 isoform as well as the increased potency of vesicle rupture that fibrillar species likely possess, although we cannot separate these properties using this assay. Current hypotheses of intercellular prion-like  $\alpha$ -syn templating postulate that either freely diffusible or vesicular  $\alpha$ -syn in exosomes may be shared between cells to mediate their pathological spread (8). Our study raises the possibility that the principal determinant or rate-limiting step for internalization is the attachment of the misfolded protein to the cell exterior, and this step may be relevant for therapeutic blockade.

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# Effects of Serine 129 Phosphorylation on α-Synuclein Aggregation, Membrane Association, and Internalization

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