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Lewy-like aggregation of α -synuclein reduces PP2A activity in vitro and in vivo

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Abstract

α -Synuclein (α -Syn) is a chaperone-like protein that is highly implicated in Parkinson's disease (PD) as well as in Dementia with Lewy Bodies (DLB). Rare forms of PD occur in individuals with mutations of α -Syn or triplication of wild type α -Syn, and in both PD and DLB the intraneuronal inclusions known as Lewy bodies contain aggregated α -Syn that is highly phosphorylated on serine 129. In neuronal cells and in the brains of α -Syn overexpressing transgenic mice, soluble α -Syn stimulates the activity of protein phosphatase 2A (PP2A), a major serine/threonine phosphatase. Serine 129 phosphorylation of α -Syn attenuates its stimulatory effects on PP2A and also accelerates α -Syn aggregation, however, it is unknown if aggregation of α -Syn into Lewy-bodies impairs PP2A activity. To assess for this, we measured the impact of α -Syn aggregation on PP2A activity in vitro and in vivo. In cell free assays, aggregated α -Syn had ~50 % less PP2A-stimulatory-effects than soluble recombinant α -Syn. Similarly in DLB and α -Syn triplication brains, which contain robust α -Syn aggregation with high levels of serine 129 phosphorylation, PP2A activity was also ~50% attenuated. As α -Syn normally stimulates PP2A activity, our data suggest that overexpression of α -Syn or sequestration of α -Syn into Lewy bodies has the potential

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Disclosure Statement

The authors declare no potential conflict of interest with regard to financial, personal or other relationships that could have inappropriately biased this work.

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to alter the phosphorylation state of key PP2A substrates; raising the possibility that all forms of synucleinopathy will benefit from treatments aimed at optimizing PP2A activity.

Keywords

Synucleinopathy; hyperphosphorylation; dephosphorylation; phosphatase; enzymatic regulation

α -Syn is an abundant chaperone-like protein (Maroteaux et al., 1988) that contributes to brain neuroplasticity as well as to neurodegeneration (Clayton and George, 1998). α -Syn is a member of a family of proteins that also includes β - and γ -synucleins; however, α -Syn is the only synuclein that is implicated as being causative of neurodegenerative diseases. Diseases with α -Syn Lewy-like protein aggregates (Spillantini et al., 1997) are collectively referred to as synucleinopathies and these include Parkinson's disease (PD), Alzheimer's disease (AD), Dementia with Lewy Bodies (DLB), as well as Multiple System Atrophy (Galvin et al., 2001). In rare families with PD, α -Syn mutations (A30P, A53T, or E46K) (Polymeropoulos et al., 1997, Kruger et al., 1998, Zarranz et al., 2004) and multiplications (Singleton et al., 2003, Chartier-Harlin et al., 2004) have been identified. Evidence from both humans and animal models support the notion that α -Syn aggregation confers a toxic gain of function in disease states (Rajagopalan and Andersen, 2001, Eriksen et al., 2003), which includes the finding of Lewy body pathology in > 90% of sporadic PD cases (Lee and Trojanowski, 2006); however, the molecular mechanisms associated with Lewy body formation and the resulting neuronal dysfunction associated with synucleinopathies remain unclear.

We and others have previously demonstrated that α -Syn contributes to normal cellular physiology (Perez and Hastings, 2004, Sidhu et al., 2004, Geng et al., 2010). For instance, α -Syn interacts with and modulates the activity of key enzymes including the catalytic subunit of protein phosphatase 2A (PP2A) (Peng et al., 2005, Lou et al., 2010), a phosphatase that contributes broadly to normal brain function (Sim, 1991, Sim et al., 2003).

PP2A is a trimeric protein composed of a structural A subunit that dimerizes with the catalytic C subunit (PP2Ac), that then bind to particular B subunits. The substrate specificity of PP2A appears to be conferred by the regulatory B subunits (Cegielska et al., 1994, Csontos et al., 1996) which affect PP2A targeting to particular intracellular sites such as microtubules (Sontag et al., 1995, McCright et al., 1996) and mitochondria (Ruvolo et al., 2002). Although many PP2A holoenzymes can be formed, based on their specific B subunit composition, the enzymatic activity of PP2A is conferred solely by the catalytic PP2Ac subunit, with which α -Syn interacts (Peng et al., 2005). Among the many cellular substrates for PP2A (Lechward et al., 2001), several are critical regulators of brain function including the dopamine regulatory enzyme tyrosine hydroxylase (Leal et al., 2002, Saraf et al., 2010), the mitogen activated extracellular-regulated kinases (ERK1/2) (Letourneux et al., 2006), and the microtubule associated protein tau (Sontag et al., 1996, Sontag et al., 1999). Remarkably, all of these proteins are not only PP2A substrates but they also interact with α -Syn directly or indirectly (Jensen et al., 1999, Iwata et al., 2001, Perez et al., 2002). It is well known that tau becomes hyperphosphorylated in brain regions with low PP2A activity (Gong et al., 1993) and that in AD brain, hyperphosphorylated tau is commonly associated with a decrease in PP2A protein (Kins et al., 2001, Sontag et al., 2004, Schild et al., 2006, Deters et al., 2009). Indeed, α -Syn aggregation is associated with reduced levels of soluble α -Syn protein in DLB brain (Baba et al., 1998), and in mice the aggregation of α -Syn occurs coincident with hyperphosphorylation of the PP2A substrate tyrosine hydroxylase (Alerte et al., 2008). Furthermore, the phosphorylation of α -Syn at serine 129 is known to both stimulate α -Syn aggregation (Chen and Feany, 2005, Smith et al., 2005) and to reduce

α -Syn-associated PP2A activity (Lou et al., 2010), making studies to further elucidate the functional interplay between α -Syn aggregation and PP2A regulation an important research topic.

Herein, we explored if aggregation of α -Syn can alter PP2A activity. We evaluated the effects of soluble and insoluble recombinant α -Syn on recombinant PP2A. For in vivo assays, we compared α -Syn aggregation and PP2A activity in human control, DLB, and α -Syn triplication frontal cortex. Levels of α -Syn serine 129 phosphorylation (PSer129) were also measured by immunoblot and immunohistochemistry. Our resulting data are the first demonstration that PP2A activity is diminished in association with α -Syn aggregation both in vitro and in vivo; suggesting that in addition to a toxic gain of function, a loss of normal α -Syn function likely contributes to pathology in synucleinopathies.

Experimental Procedures

Human brains

Medial frontal cortex from control (N = 7) and DLB (N = 7) cases were obtained from the University of Pittsburgh Alzheimer Disease Research Center Brain Bank and were handled according to protocols approved by the University of Pittsburgh Institutional Review Board. The diagnosis of DLB was confirmed according to established international consortium consensus guidelines (McKeith, 2006). For α -Syn triplication analysis, postmortem frontal cortex was obtained from one affected and three unaffected age matched individuals from a single family that had ample tissue for these extensive studies. The extremely rare tissues were generously provided by Drs. Mark Cookson and Andrew Singleton at NIH. To measure the effect of α -Syn aggregation on PP2A activity, we calculated the ratio of PP2A activity in the insoluble fraction relative to PP2A activity of the soluble fraction for each sample, which allowed each subject to serve as their own control. As data for the control group were similar, we combined their results for graphical comparison to the data for the affected individual. All tissues were deidentified to allow evaluation in a blinded manner. Demographics of all cases are shown in Table 1.

Immunostaining

Deparaffinized medial frontal cortical sections of control and DLB brain were permeabilized and blocked for 1 hr in PBS containing 5% BSA, 10% goat serum, 0.1% glycine and 0.05% Triton-X-100, then incubated overnight at 4°C in primary antibodies α -Syn (610786); α -Syn PSer129 (11A5); PP2Ac (05-421). Secondary antibodies were conjugated to Cy3 (Jackson ImmunoResearch Labs) or Alexafluor 488 (Invitrogen). Protein aggregation on human brain tissue was measured after proteinase K (PK; Invitrogen) treatment of sections encircled by Pap-Pen (Polysciences, Inc.; Warrington, PA), rewet in 1 × PBS, and digested in 2.5 g/ml PK in 0.1 M Tris-HCl buffer, 0.005 mM EDTA, pH 7.5 for 15–30 min at room temperature. Sections were immunostained, light protected, and stored at 4°C prior to FluoView™ (Olympus) quantitative analysis as previously described (Alerte et al., 2008).

Immunoblots

Protein samples (20 or 25 μ g) were separated by SDS-PAGE on 12% Tris-Glycine gels and transferred to nitrocellulose. Equivalent sample loading was confirmed by Ponceau S staining and β -actin immunoblotting as an internal control. Membranes were blocked in 10% milk-PBS and incubated overnight at 4°C in primary antibody. Antibodies utilized for α -Syn were sc-7011R (Santa Cruz); 610786 (BD Biosciences) and PSer129 α -Syn clone 11A5 (gift of J Anderson, Elan Pharmaceutical). PP2Ac antibodies included a full-length PP2Ac antibody, FL309 (sc-14020, Santa Cruz) and two C-terminal PP2Ac antibodies (1D6, 05-421, Millipore/Upstate and sc-6110, Santa Cruz). PP2A signal was equivalent for blots

probed with 1D6, sc-6110, and FL309 antibodies (Fig 1). β -actin A5441 antibody was from Sigma-Aldrich. Infrared signal was obtained using anti-mouse, anti-goat, or anti-rabbit secondary antibodies coupled to IgG IRDye680 or IgG IRDye800 (1:5000 – 1:10,000; Rockland Immunochemicals) and imaged with an Odyssey system (LiCor Biosciences). Signal was quantified within a linear range as before (Perez et al., 2002, Peng et al., 2005). We often see doublet bands of PP2A on blots from well resolved gels, which may represent molecular weight differences associated with posttranslational modification (e. g. Fig 1B).

PP2A assays of brain tissue

Using well established protocols (Cohen et al., 1989, Sontag et al., 2004, Lou et al., 2010) frozen brain was thawed, homogenized in imidazole buffer [20 mM imidazole-HCl, 2 mM EDTA, 2 mM EGTA plus protease inhibitors] at 4°C on ice and centrifuged to remove particulates, followed by free phosphate removal on Microspin™ G-25 columns (GE Healthcare). Supernatant aliquots were incubated in pNPP buffer [50mM Tris-HCl, pH 7.0, 0.1mM CaCl₂] with KRpTIRR phosphopeptide for 10 min at 30°C. Triplicate samples were assayed spectrophotometrically at 650 nm relative to known standards using malachite green assay (17–127; Millipore/Upstate) on 2 – 6 independent occasions. Specificity controls from Tocris Bioscience (Ellisville, MO, USA) included 50 nM protein-phosphatase-inhibitor 2 (inhibits PP1), 10 nM okadaic acid (this dose inhibits PP2A, PP4, PP5) and 100 nM fostriecin (this dose inhibits PP2A and PP4) (Swingle et al., 2007). Recombinant Proteins: α -Syn (generously provided by Dr. John Rosenberg, University of Pittsburgh) was incubated with the recombinant PP2A catalytic subunit (PP2Ac; Cayman Chemicals, Ann Arbor, MI) or PP2Aac (both the A and C subunits, Millipore-Upstate, Billerica, MA) for 30 min at 4°C in pNPP buffer, then in the presence of phosphopeptide KRpTIRR at 30°C for 10 min as described above. Data from duplicate or triplicate samples were evaluated on 3 – 5 independent occasions for each data set.

α -Syn sequential extraction

To obtain α -Syn aggregates from human brain, we used well established methods (Waxman and Giasson, 2008) in which medial frontal cortices were homogenized in 3 volumes of ice cold high-salt (HS) buffer [50 mmol/L of Tris, 750 mmol/L of NaCl, 5 mmol/L of EDTA with 10 ug/ml leupeptin, 10 ug/ml aprotinin, 1 mM benzamidine and 1 mM AEBSF, 50 mmol/l of sodium fluoride, 1 mmol/L of sodium orthovanadate, and 1 μ mol/L of okadaic acid]. Samples were sedimented at 100,000 $\times$ g for 20 min at 4°C, and supernatants removed and stored at –80°C. Pellets were re-homogenized in the following buffers with the centrifugation steps, as described above, between each extraction step. Buffer 2 was HS buffer containing added 1% Triton X-100 (HS/Triton), buffer 3 was RIPA (50 mmol/L of Tris, 150 mmol/L of NaCl, 5 mmol/L of EDTA, 1% NP 40, 0.5% sodium deoxycholate, and 0.1% SDS), and buffer 4 was SDS/Urea (8 mol/L of urea, 2% SDS, 10 mmol/L Tris; pH 7.5). Prior work has demonstrated that all α -Syn bands on SDS/Urea blots are aggregate species, including the 19 kDa band (Waxman and Giasson, 2008). The concentration of protein for each sample was confirmed using the bicinchoninic acid assay (BCA, Thermo Pierce). Laemmli buffer was added, then samples were heated to 95°C for 5 min prior to SDS-PAGE followed by immunoblot, with the exception of the SDS/Urea samples, which were not boiled prior to gel loading as delineated by the protocol. Human samples were handled in a biosafety cabinet by individuals wearing appropriate personal protection garb and with equipment decontamination accomplished using 10% bleach.

Dialysis of samples prior to PP2A assay

Two fractions from serially extracted α -Syn triplication and control brains were assessed for PP2Ac activity. The HS (Soluble) and SDS/Urea (Insoluble) extracts were prepared as above then dialyzed against pNPP buffer [50mM Tris-HCl, pH 7.0, 0.1mM CaCl₂] by

floating each sample on a 0.025 μm Type VS membrane (Millipore) for the equilibration step. Briefly, a hydrophobic barrier was created by drawing a small circle with a Pap-Pen on the surface of a membrane. Samples were then gently pipetted onto the membrane and dialyzed against 0.5 L of pNPP buffer with gentle stirring 45 min at room temperature. This dialysis method allows complete buffer exchange with ~98% protein recovery (Marusyk and Sergeant, 1980), as confirmed by BCA assay. For quality assurance we performed control experiments in which recombinant PP2A was suspended in HS or SDS/Urea buffers, incubated, then dialyzed as described above. Both the HS and SDS/urea PP2A samples sustained high levels of enzymatic activity after dialysis, confirming both the efficiency of HS and SDS/Urea buffer removal and the active state of PP2A prepared in this way (Fig. 4B).

Recombinant α -Syn aggregation

WT human recombinant α -Syn was aggregated using protein prepared in 100 mM sodium acetate, pH 7.4, at 5 mg/ml followed by constant agitation for 48 hr at 37°C as previously described (Giasson et al., 1999). α -Syn aggregates were precipitated by centrifugation at $100,000 \times g$ for 20 min, with insoluble α -Syn in the pellet (P) and soluble α -Syn (S) remaining in the supernatant. For Coomassie stained gels, Laemmli buffer was added to S and P fractions prior to heating to 100°C for 15 min. α -Syn samples were resolved by 12% Tris-Glycine SDS-PAGE followed by fixation and staining with Coomassie Brilliant Blue R-250. Stained gels were scanned and aggregation was quantified from images using ImageQuant (Molecular Dynamics, GE Healthcare Life Sciences). The percentage of aggregated α -Syn was calculated using the formula $[P/(P+S)]/100$. Volumes were measured before transferring soluble supernatants (S) to new tubes after which the insoluble pellets (P) were resuspended in 100 mM sodium acetate (pH 7.4) in a volume equal to that of the supernatant sample and equal volumes of each sample were then evaluated for PP2A activity.

Statistics

Independent sample Student's *t* tests and ANOVA with Tukey-Kramer posthoc analyses were performed as appropriate to the data, using InStat (Graph Pad Software). Data were considered significant at $P < 0.05$. Results were confirmed in a minimum of 2 – 3 independent experiments using duplicate or triplicate samples, in which data represent mean $\pm$ standard error of the mean (SEM).

Results

Aggregated wild type α -Syn is a less effective stimulator of PP2A activity

As previously demonstrated, α -Syn aggregates in Lewy bodies, and in neuronal cells α -Syn binds to and stimulates PP2A activity (Peng et al., 2005), however, no one has assessed if α -Syn aggregation might impair α -Syn-mediated stimulation of PP2A. To directly assess for PP2A effects, and to exclude contributions of related serine/threonine phosphatases such as PP1, PP4, or PP5, we used recombinant PP2A and recombinant α -Syn. Soluble recombinant PP2A was assayed for its ability to release phosphate from a phosphorylated peptide substrate. Baseline values were obtained with PP2A alone, and the effect of α -Syn was assessed by adding either soluble or aggregated wild type (WT) α -Syn to PP2A. We prepared soluble and insoluble α -Syn using well established methods (Giasson et al., 1999). Briefly, after incubation α -Syn was collected by centrifugation with soluble α -Syn in the supernatant (S) and insoluble α -Syn in the pellet (P). Soluble and aggregated α -Syn were evaluated by immunoblot (Fig 2A) in which high molecular species of α -Syn were present in the insoluble pellet "P" along with a 19 kDa α -Syn aggregate as previously demonstrated (Giasson et al., 1999). Using this method, ~65% of recombinant WT α -Syn aggregated in

our preparations and was present in the insoluble protein pellet (Fig 2B). When we assayed the activity of recombinant PP2A in response to α -Syn (as detailed in Experimental Procedures), we noted that at baseline PP2A without added α -Syn efficiently cleaved PO₄ from the peptide substrate, while addition of soluble WT α -Syn significantly stimulated PP2A activity, and insoluble WT α -Syn produced ~50% less stimulatory effect on PP2A activity (Fig 2C; $P < 0.001$, ANOVA) in cell free in vitro assays. These findings and our prior animal data (Alerte et al., 2008) suggest that aggregated α -Syn is less able to stimulate PP2A activity in vivo and in vitro. To assess this in human brain samples we obtained pathologically-confirmed DLB medial frontal cortex and equivalent tissue from elderly control brains.

PP2A activity is lower in DLB frontal cortex compared to controls

Having demonstrated that aggregated recombinant α -Syn was ~50% less able to stimulate soluble recombinant PP2A activity in vitro, we next assessed the impact of α -Syn aggregation on phosphatase activity in DLB brains. Levels of PP2A protein were similar on immunoblots prepared using identical microgram amounts of total protein from control and DLB frontal cortex, as further confirmed by an actin loading control (Fig 3A). When we measured phosphatase activity using established methods (Cohen et al., 1989, Sontag et al., 2004, Lou et al., 2010), we found that DLB frontal cortex had ~ 50% less phosphatase activity compared to controls (Fig 3B; t-test, $P < 0.01$). For specificity we used the PP2A inhibitor fostriecin (Walsh et al., 1997), which does not inhibit PP1, PP2B, or PP5 at low nM concentrations (Swingle et al., 2007). Fostriecin produced > 50% inhibition of phosphate release from human cortical samples (0 nM, 2.57 ± 0.26 pMol PO₄/min/ug protein; 100 nM; $0.72 \pm .10$ pMol PO₄/min/ug protein; $P < 0.05$), not unlike the ~50% decrease noted in response to aggregated α -Syn in cell free assays (Fig 2C) or the ~50% decrease in phosphatase activity noted in DLB samples (Fig 3B). Thus, these data confirm that PP2A activity is impaired in brain regions with Lewy-like α -Syn aggregates (Fig 3C, 3D). Even so, 100 nM fostriecin can also inhibit PP4, but not PP5, raising the possibility that in addition to PP2A, PP4 activity may also be affected in DLB brains.

To delineate the levels of soluble and insoluble α -Syn in control and DLB frontal cortices, we sequentially extracted proteins using published methods that produced four fractions, three of which are soluble and one insoluble (Waxman and Giasson, 2008). Using ultracentrifugation and a defined series of buffers, pellets were collected then re-extracted after each centrifugation step. For soluble fractions we used high salt buffer (HS), followed by high salt + Triton X-100 (HS/Triton), and then RIPA buffer. The RIPA pellet was re-extracted in SDS/Urea to yield insoluble proteins that accumulate even in normal elderly brains (Woltjer et al., 2009). Quantitative densitometry of immunoblots allowed us to compare α -Syn signals in the soluble and insoluble fractions, with resulting data being expressed in arbitrary units (A.U.). Total α -Syn signal was similar in the soluble HS fractions of control ($27,079 \pm 2205$ A.U.) and DLB frontal cortex ($26,568 \pm 2197$ A.U.) (Fig 3C, top left blots; $P = 0.88$, t test). The finding of similar total soluble α -Syn levels in control and DLB cortex HS fractions is not unexpected because brain α -Syn levels are known to increase in aged humans and aged non-human primates (Chu and Kordower, 2007).

When the HS pellets were re-extracted in HS/Triton buffer to release membrane-associated soluble proteins, significantly less α -Syn was present in DLB brain ($13,378 \pm 1845$ A.U.) compared to controls ($20,971 \pm 551$ A.U.) (Fig 3C, top, second from left; $P = 0.017$, t test), suggesting that DLB brain has less soluble α -Syn associated with membranes. Extraction of the HS/Triton pellets in RIPA buffer yielded similar low levels of soluble α -Syn from DLB (5103 ± 244 A.U.) and control frontal cortices (5529 ± 591 A.U.) (Fig 3C, top, second from right; $P = 0.54$, t test), confirming the efficiency of soluble α -Syn extraction in prior

fractions. The final extraction of insoluble aggregated proteins was performed using SDS + Urea buffer (SDS/Urea) on the RIPA pellets, which yielded 10 fold higher levels of insoluble α -Syn from DLB brain ($107,983 \pm 25415$ A.U.) compared to control frontal cortices ($11,112 \pm 3661$ A.U.), consistent with the abundant Lewy body pathology confirmed by neuropathological diagnosis on our DLB samples (Fig 3C, top right; $P < 0.019$, t test).

Others have demonstrated excess α -Syn with high levels of PSer129 in cortices with Lewy body pathology (Saito et al., 2003). To assess PSer129 α -Syn levels in our samples, we compared parallel HS, HS/Triton, RIPA, and SDS/Urea immunoblots from a subset of samples in which the larger amount of tissue necessary for these more extensive analyses was available. In control frontal cortex, PSer129 α -Syn signal was not apparent on immunoblots from any of the extracts. In stark contrast, PSer129 α -Syn signal was measurable in both the soluble and insoluble fractions of frontal cortex from DLB brains (Fig 3C, bottom row). Quantitative densitometry of PSer129 signals also confirmed significantly higher levels of PSer129 α -Syn in DLB frontal cortex from the following fractions: soluble HS ($P = 0.014$, t test), soluble HS/Triton ($P = 0.028$, t test), and insoluble SDS/Urea fractions ($P = 0.034$, t test).

To confirm α -Syn aggregation and PSer129 levels in frontal cortex from control and DLB cases, we pretreated some samples with proteinase K followed by immunostaining and confocal microscopy. Large α -Syn aggregates were common in DLB brain (Fig 3D, top left panel) while only small punctate α -Syn aggregates were ever seen in aged matched control brain (Fig 3D, bottom left panel). When we measured PSer129 immunoreactivity, control brain had faint staining (Fig 3D, bottom middle) compared to the stronger more extensive immunoreactivity in the DLB brain (Fig 3D, top middle). Quantitative assessment of PSer129 signals using FluoViewTM allowed us to generate signal intensity profiles from equal sized representative fields of control and DLB brain in which we noted at least three fold more PSer129 signal in DLB frontal cortex (2079.3 ± 27.3 pixels) compared to controls (682.1 ± 10.4 pixels) ($t = 47.9$, $P < 0.0001$; Fig 2D, right side). This finding matches our immunoblot data (Fig 3C) and supports findings by others of robust PSer129 immunoreactivity in regions of PD and DLB brain with significant α -Syn aggregation (Fujiwara et al., 2002, Anderson et al., 2006). However, ours are the first data showing that PP2A activity is also significantly impaired in regions of DLB brain with high levels of α -Syn aggregation and robust α -Syn serine129 phosphorylation. To assess for potential effects of α -Syn aggregation on phosphatase activity in PD, we also evaluated frontal cortex from members of a rare α -Syn triplication family (Singleton et al., 2003). The demographics of unaffected and affected members of this family with adequate tissue available for our extensive biochemical analysis are shown in Table 1.

PP2A activity is reduced in parallel with increased α -Syn aggregation in α -Syn triplication brain

To compare soluble to insoluble fractions, we serially extracted proteins from frontal cortex of unaffected controls and α -Syn triplication brain using the same buffers and extraction method used for DLB brain above, followed by dialysis to remove buffers as described in Experimental Procedures. We were limited to a small sample size for these experiments by the required amount of brain tissue available from this rare PD family. We assessed α -Syn and PP2A levels by immunoblot and PP2A activity in the dialyzed HS soluble fraction and SDS/Urea insoluble fraction. α -Syn protein in the triplication case was 2-fold higher than controls (Fig 4A, Soluble), as previously reported for this same family (Singleton et al., 2003). Total PP2A levels varied in the soluble fractions of frontal cortex, with less PP2A being noted in equal amounts of protein extracted from triplication brain compared to control (Fig 4A, Soluble). A slight increase in total PP2A was noted in equal amounts of

extract of insoluble fraction from triplication brain (Fig 4A, Insoluble), suggesting that some PP2A may get sequestered with α -Syn in Lewy bodies of PD.

To control for variability, PP2A activity data were normalized to PP2A protein levels for each condition. We first confirmed that PP2A signal on immunoblots was similar using different anti-PP2A catalytic subunit antibodies for normalizing PP2A (Fig 1). In control and α -Syn triplication brain, PP2A levels were more similar in the insoluble fractions (Fig. 4A, Insoluble) with more total α -Syn again being observed on the triplication immunoblot. Among the four cases, the triplication alone had measurable levels of PSer129 α -Syn in the insoluble fraction (Fig 4A, Insoluble). In fact, no measurable PSer129 signals were seen in the soluble fractions of any cortical sample (not shown).

As an additional control, we assayed recombinant PP2A that had been prepared in HS or SDS/Urea buffer followed by dialysis. PP2A activity from the HS sample was set as the control, which allowed us to confirm the recovery of highly active PP2A from both buffers, and no significant difference between conditions for recombinant PP2A prepared with this method (Fig 4B), confirming that this technique is valuable for enzymatic assays from human brain samples.

We then compared PP2A activity from control and triplication cases. The activity of PP2A in the HS soluble fraction was significantly greater in α -Syn triplication brain (1.113 ± 0.01 pmol PO₄/min/ μ g protein) than in controls (0.67 ± 0.04 pmol PO₄/min/ μ g protein), as might be expected for samples with two fold more soluble α -Syn ($P < 0.001$, t test). In contrast, insoluble α -Syn from triplication brain, which had measurable α -Syn PSer129 levels (Fig. 4A, Insoluble, right side), also had significantly less PP2A activity compared to controls (Fig. 4C; $P < 0.001$, t test). This finding strongly suggests that aggregated α -Syn has significantly less ability to stimulate PP2A activity, much like our findings with recombinant α -Syn and PP2A (Fig 2).

Discussion

There is rising interest in the role of α -Syn aggregation in neurodegeneration. One theory states that α -Syn aggregation impairs neuronal activity (Cookson and van der Brug, 2008) and others suggest that protofibrillar oligomers of α -Syn are toxic (Goldberg and Lansbury, 2000, Lashuel and Lansbury, 2006). We have found that in vivo α -Syn aggregation is associated with hyperphosphorylation of the key PP2A substrate, tyrosine hydroxylase, in dopaminergic neurons (Alerte et al., 2008). In vitro studies have demonstrated that recombinant α -Syn also readily polymerizes into amyloidogenic fibrils that are structurally similar to those found in human brain (Conway et al., 1998, Hashimoto et al., 1998, Giasson et al., 1999, Narhi et al., 1999). Taken together the data suggest that α -Syn aggregation has the potential to impair PP2A activity, although that possibility had not been previously explored. In the present study we assessed PP2A activity as a function of α -Syn aggregation in various model systems. Our findings with recombinant proteins demonstrated a direct effect of α -Syn aggregation on PP2A activity impairment. Using DLB brain and α -Syn triplication brain we noted a loss of PP2A activity in association with α -Syn aggregation, supporting the hypothesis that PP2A activity appears to be attenuated in response to α -Syn aggregation, a process that can be accelerated by α -Syn Ser129 phosphorylation.

Hansen and colleagues long ago demonstrated that DLB frontal cortex contains abundant Lewy body pathology (Hansen et al., 1993) and our data (Fig 3) concur with their findings. More recent work has shown that, as previously anticipated (Spillantini et al., 1997) α -Syn is the major protein component of LBs (Anderson et al., 2006) indicating that evaluation of frontal cortex for changes associated with α -Syn aggregation should be instructive.

We measured PP2A activity in frontal cortex of control and DLB brains using established methods with specificity controls (Cohen et al., 1989, Sontag et al., 2004, Lou et al., 2010) and found that DLB brains, which contain significantly more aggregated α -Syn (Fig 3C and 3D) also had significantly less PP2A activity than controls. Because α -Syn is a normal activator of PP2A, it is therefore likely that as α -Syn becomes sequestered in Lewy bodies; levels of soluble functional α -Syn become diminished, causing PP2A to be less active in brain regions harboring synucleinopathy. This is also supported by our findings from α -Syn triplication frontal cortex where there was a huge loss of PP2A activity compared to the soluble fraction of the identical tissue (Fig 4), even though α -Syn protein content was high in both soluble and insoluble fractions. Taken together, the data imply that a reduction in soluble α -Syn and/or an increase in α -Syn P_{Ser129} levels is sufficient to impair PP2A activity in brains with synucleinopathy, and in DLB brains it appears that it may be the combination of α -Syn aggregation and elevated P_{Ser129} levels that act to diminish PP2A activity. These findings have implications for hyperphosphorylation of PP2A substrates, such as tau protein, which is consistently hyperphosphorylated in AD brain as well as in some cases of PD and DLB (Arima et al., 1999) where abundant synucleinopathy is also found. There is also a possibility that as α -Syn becomes aggregated, PP2A may remain bound to it and become sequestered in Lewy bodies, reducing the soluble pool of active PP2A in the brain. Data from the triplication brain hint at this possibility (Fig 4A, compare PP2A in S and P fractions) a possibility that we are further exploring.

It is noteworthy that the substrate KRpTIRR used in our assays can be dephosphorylated by other serine/threonine phosphatases besides PP2A, including PP4 and PP5. Furthermore, recent studies have shown that PP5 is implicated in Alzheimer's disease (Liu et al., 2005; Sanchez-Ortiz et al., 2009). Nonetheless, there are at least three lines of evidence that strongly support the role for PP2A as proposed herein: (first) our prior work measured the activity of PP2A that was immunoprecipitated with a PP2A-specific antibody, which allowed us to demonstrate that PP2A activity is enhanced by α -Syn (Peng et al., 2005); (second) our recombinant PP2A cell free assays with soluble α -Syn or aggregated α -Syn (Fig 2) gave results similar to our findings in control and DLB brains (Fig 3); (third) inhibition of phosphatase activity with fostriecin paralleled the loss of PP2A activity noted in DLB brains (Fig 3B). As previously stated, we cannot rule out a potential effect on PP4 as well, a possibility that should be further explored.

While α -Syn and the catalytic subunit of PP2A are known to interact (Peng et al., 2005), no one knows if α -Syn might also bind B subunits of PP2A to in some manner contribute to holoenzyme assembly or localization. Both α -Syn and PP2A are localized to mitochondria in neuronal cells (Perez et al., 2002, Wang et al., 2009) and B subunits of PP2A have also been identified on mitochondria including subunits B56 (Ruvolo et al., 2002) and B β 2 (Dagda et al., 2003).

We have begun exploring the mechanisms underlying α -Syn-mediated regulation of PP2A and the changes that occur with α -Syn aggregation. Preliminary data suggest that aggregated α -Syn may undergo a structural change that alters its ability to bind the PP2A catalytic subunit, which may then shift the phosphatase to a less active conformational state. In sum, this study reveals that α -Syn overexpression stimulates PP2A activity and α -Syn aggregation impairs PP2A activity, and may affect PP4 as well; implying that a gain or loss of normal α -Syn function in brains with synucleinopathy may induce phosphatase dysregulation. Importantly, novel therapies to modulate PP2A activity are being explored (Lu et al., 2009, Corcoran et al., 2010) and if successful, such treatments could benefit conditions in which levels of functional soluble α -Syn are found to vary.

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Highlights

- Alpha-synuclein (α -Syn) activates a major serine/threonine protein phosphatase, PP2A.
- Impaired PP2A activity leads to hyperphosphorylation of PP2A substrates.
- Aggregated α -Syn is a less potent activator of PP2A in cell free assays and brain.
- Overexpression of α -Syn or Lewy body formation is associated with impaired PP2A activity in brain.
- PP4 phosphatase may also be affected.

Figure 1. Multiple PP2A antibodies label the catalytic subunit of brain PP2A equally well
 The use of different antibodies for normalizing our PP2A data made it essential to determine their ability to label total PP2Ac from brain extracts. **A.** Immunoblot of 20 μ g total protein from two different samples of mouse cortex was reacted with sc-6110 (rabbit polyclonal antibody) and 1D6 (mouse monoclonal antibody), both of which label the 36 kDa PP2A band equally as visualized using Odyssey by LiCor®. Secondary antibodies were anti-rabbit IRDye 800 (green) for labeling sc-6110, and anti-mouse IRDye 680 (red) to label 1D6, which creates a yellow signal when merged, demonstrating nearly complete overlap of PP2Ac signals. **B.** Immunoblot of 20 μ g total protein from two different samples of human frontal cortex labeled with a full length 309 amino acid PP2Ac antibody sc-14010 (rabbit polyclonal), and with antibody 1D6 (mouse monoclonal). Secondary antibodies were anti-rabbit IRDye 680 (red) and anti-mouse IRDye 800 (green) which produce a yellow image when merged. These results confirm essentially identical labeling of the PP2A catalytic subunit with all antibodies used for evaluations.

Figure 2. Aggregated wild type recombinant α -Syn is less able to stimulate PP2A activity
 (A) Representative Coomassie stained gel showing WT recombinant human α -Syn in the soluble (S) and insoluble pellet (P) fraction. (B) The percent of α -Syn accumulating in the insoluble pellet was calculated as described in Experimental Procedures. (C) PP2A activity of soluble and insoluble α -Syn samples demonstrate a significant loss in activity associated with α -Syn aggregation. Histogram represents the mean $\pm$ SEM of duplicate samples from four independent experiments. ** P < 0.01, *** P < 0.001, Student's t test.

Figure 3. DLB frontal cortex has abundant insoluble α -Syn with highly phosphorylated Ser129 and lower PP2A activity

(A) Representative control and DLB frontal cortex samples have similar total PP2Ac. Actin serves as a loading control. (B) Despite similar levels of PP2A protein, PP2A enzymatic activity is significantly lower in DLB frontal cortex. (C) Identical protein amounts from sequential extractions of frontal cortex show similar total α -Syn levels in the soluble fractions on immunoblots of all cases; however, much more total α -Syn is seen in DLB insoluble fractions. All fractions of DLB frontal cortex have measurable levels of PSer129 α -Syn while control fractions have none. (D) Tissues pretreated with proteinase K demonstrate abundant α -Syn aggregates and PSer129 α -Syn staining in DLB frontal cortex compared to small punctuate aggregates with very faint PSer129 α -Syn staining in control frontal cortex. Scale bar, 50 μ m. Data represent the mean $\pm$ SEM of 7 DLB and 7 control cases (N = 14). ** P < 0.01, Student's t test.

Figure 4. α -Syn aggregation reduces PP2A activity in α -Syn triplication brain

A. Representative immunoblots from soluble (left side) and insoluble (right side) fractions of control (Cont) and α -Syn triplication (Trip) frontal cortex. Actin serves as a loading control. α -Syn is increased in triplication frontal cortex, which also has measurable levels of P Ser129 α -Syn, while control brain does not. **B.** Control experiments using equal amounts of recombinant PP2A prepared in high salt (HS) or SDS/Urea buffer, then dialyzed against pNPP buffer, were assayed for PP2A activity. Activity of PP2A was equivalent in repeated experiments performed on triplicate samples. HS served as the control condition. **C.** To demonstrate PP2A effects resulting from α -Syn aggregation, we plotted the ratio of insoluble to soluble PP2A activity, which reveals ~50% less PP2A activity in triplication frontal cortex despite the higher total α -Syn levels (Total SYN). Student's t-test, $P < 0.001$. Data represent the mean $\pm$ SEM of three independent experiments using duplicate samples.

Table 1

Demographics of subjects from whom postmortem medial frontal cortical samples were obtained. All diagnoses were confirmed using established neuropathological assessment methods.

	AGE	PMD (HRS)	SEX
CONTROLS	62.4 ($\pm$ 10.8)	9.4 ($\pm$ 5.9)	(6 M, 1 F)
DLB	74.4 ($\pm$ 6.4)	5.6 ($\pm$ 3.5)	(3 M, 4 F)
CONTROLS	45 ($\pm$ 5.5)	9	(2M, 1F)
SYN TRIPLICATION	51	9	F