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LEWY-LIKE AGGREGATION OF α-SYNUCLEIN REDUCES PROTEIN PHOSPHATASE 2A 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 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.

Key words: synucleinopathy, hyperphosphorylation, dephosphorylation, phosphatase, enzymatic regulation.

α-synuclein (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; Krüger 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) and binds to particular B subunits. The substrate specificity of PP2A appears to be conferred by the regulatory B subunits (Cegielska et al., 1994; Csortos 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

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Abbreviations: AD, Alzheimer's disease; DLB, dementia with Lewy bodies; EDTA, Ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; PD, Parkinson's disease; PP2A, protein phosphatase 2A; α-Syn, α-synuclein.

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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, 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 (National Institutes of Health, Bethesda, MD, USA). 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 its 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 de-identified to allow evaluation in a blinded manner. Demographics of all cases are shown in Table 1.

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 (hr)	Sex
Controls	62.4 (±10.8)	9.4 (±5.9)	(6 M, 1 F)
DLB	74.4 (±6.4)	5.6 (±3.5)	(3 M, 4 F)
Controls	45 (±5.5)	≤9	(2 M, 1 F)
SYN Triplication	51	≤9	(1 F)

Immunostaining

Deparaffinized medial frontal cortical sections of control and DLB brain were permeabilized and blocked for 1 h 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, West Grove, PA, USA) or Alexa Fluor 488 (Invitrogen, Life Technologies, Grand Island, NY, USA). Protein aggregation on human brain tissue was measured after proteinase K (PK; Invitrogen) treatment of sections encircled by Pap-Pen (Polysciences, Inc., Warrington, PA, USA), 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 before FluoView™ (Olympus, Center Valley, PA, USA) quantitative analysis as previously described (Alerte et al., 2008).

Immunoblots

Protein samples (20 or 25 μg) 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 used for α-Syn were sc-7011R (Santa Cruz Biotechnologies, Santa Cruz, CA, USA); 610786 (BD Biosciences, San Jose, CA, USA), and PSer129 α-Syn clone 11A5 (gift of J Anderson, Elan Pharmaceutical, South San Francisco, CA, USA). PP2Ac antibodies included a full-length PP2Ac antibody, FL309 (sc-14020, Santa Cruz), and two C-terminal PP2Ac antibodies (1D6, 05-421, Millipore/Upstate, Billerica, MA, USA 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, Boyertown, PA, USA) and imaged with an Odyssey system (LiCor Biosciences, Lincoln, NB, USA). 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

Brain tissues. 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 MicrospinTM G-25 columns (GE Healthcare, Waukesha, WI, USA). Supernatant aliquots were incubated in pNPP buffer [50 mM Tris–HCl, pH 7.0, 0.1 mM $CaCl_2$] with KRpTIRR phosphopeptide for 10 min at 30 °C. Triplicate samples were