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Induction of the Immunoproteasome Subunit Lmp7 Links Proteostasis and Immunity in α-Synuclein Aggregation Disorders

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ABSTRACT

Accumulation of aggregated α-synuclein into Lewy bodies is thought to contribute to the onset and progression of dopaminergic neuron degeneration in Parkinson's disease (PD) and related disorders. Although protein aggregation is associated with perturbation of proteostasis, how α-synuclein aggregation affects the brain proteome and signaling remains uncertain. In a mouse model of α-synuclein aggregation, 6% of 6215 proteins and 1.6% of 8183 phosphopeptides changed in abundance, indicating conservation of proteostasis and phosphorylation signaling. The proteomic analysis confirmed changes in abundance of proteins that regulate dopamine synthesis and transport, synaptic activity and integrity, and unearthed changes in mRNA binding, processing and protein translation. Phosphorylation signaling changes centered on axonal and synaptic cytoskeletal organization and structural integrity. Proteostatic responses included a significant increase in the levels of Lmp7, a component of the immunoproteasome. Increased Lmp7 levels and activity were also quantified in postmortem human brains with PD and dementia with Lewy bodies. Functionally, the immunoproteasome degrades α-synuclein aggregates and generates potentially antigenic peptides. Expression and activity of the immunoproteasome may represent testable targets to induce adaptive responses that maintain proteome integrity and modulate immune responses in protein aggregation disorders.

1. Introduction

Cells have developed protein homeostasis networks to maintain proper cellular function and combat potentially toxic protein aggregation [1–3]. Failure to sustain proteostasis upon protein aggregation may contribute to the pathogenesis of several neurodegenerative diseases of aging such as Parkinson's disease, Alzheimer's disease and Amyotrophic lateral sclerosis [1–3]. These diseases are characterized by progressive misfolding and aggregation of proteins and ultimately neuron death.

However, it remains unclear how intracellular aggregation of proteins leads to neuron dysfunction and death. Studies in cellular model systems have shed some light on the pathological mechanisms of endogenous protein aggregation. Dynamic changes in the proteome of cultured cells following intracellular aggregation of artificial synthetic proteins indicated that the formation of amyloid-like aggregates attracted several interacting proteins, which were functionally linked to protein synthesis and quality control [4]. These findings were recently expanded to show that not only artificial synthetic proteins enriched in β-sheet structure but also fragments of mutant huntingtin and TAR DNA-binding protein 43 (TDP-43) peptides, which aggregate in

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human diseases, cause disturbances in the proteome by interfering with nuclear-cytoplasmic protein and RNA transport [5]. Collectively, these studies highlight the impact of protein aggregation on the proteome and proteostasis, and provide the intriguing hypothesis that the reallocation of cellular resources to combat changes in the proteome upon endogenous protein aggregation leads to dysfunction and ultimately neuron death.

To explore, for the first time, protein aggregation induced changes in the proteome and signaling through phosphorylation *in vivo* we capitalized on the development of a mouse model of α-synuclein aggregation and the use of quantitative mass spectrometry (MS)-based proteomic technologies. α-Synuclein is a 140 amino acid protein that is predominantly localized to vesicles in pre-synaptic terminals [6–8] participating in the regulation of neurotransmitter release and synaptic plasticity [9–11]. Several point mutations, as well as multiplications, of the α-synuclein gene are associated with familial Parkinson's disease (PD) [12–14]. Moreover, highly organized amyloid-like fibrils and non-amyloid amorphous aggregates of non-mutant α-synuclein are deposited into Lewy bodies, cytoplasmic inclusions that serve as histopathological hallmarks of sporadic PD and other related neurodegenerative disorders [15–17].

To investigate the pathological mechanisms of α-synuclein aggregation, Luk et al. developed a mouse model of α-synuclein aggregation [18]. In this model, unilateral injection of preformed fibrils (PFFs) of recombinant wild type mouse α-synuclein into the striatum of non-transgenic mice induces progressive aggregation of endogenous α-synuclein, first in regions proximal to the injection site 30 days post injection (dpi) with further involvement of distally interconnected regions by 90 and 180 dpi. Injected mice developed significant dopaminergic neuron degeneration and impaired balance and motor coordination at 180 dpi [18,19]. Importantly, degeneration and α-synuclein inclusions within the nigrostriatal dopaminergic system are confined to the injected side and absent in the non-injected contralateral side of the brain at 180 dpi. Additionally, injection of PFFs into α-synuclein null (*Snca*$^{-/-}$) mice fails to induce these effects, indicating that endogenous α-synuclein is required for aggregation and dopamine neuron degeneration [18]. This PFF injection model has been reproduced in mice, rats and non-human primates [20–24]. Therefore, this model (both wild type and *Snca* $^{-/-}$ injected mice) provides an opportunity for *in vivo* study of quantitative changes in the proteome upon aggregation of α-synuclein using MS-based proteomics and phosphoproteomics when used in combination with Stable Isotope Labeling in Mammals (SILAM) [25].

2. Methods

2.1. Animals

Wild type female, 2–3 month old, C57BL6/C3H mice were obtained from the Jackson Laboratories (Bar Harbor, ME). *Snca*$^{-/-}$ mice were maintained on a C57BL6 background. ^{13}C-Stable Isotope Labeling in Mammals (SILAM) mouse brain tissue (C57BL6 female, L-Lysine-^{13}C6, 97%) was purchased from Cambridge Isotope Laboratories, Inc. All housing, breeding, and procedures were performed according to the NIH Guide for the Care and Use of Experimental Animals and approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC).

2.2. Stereotaxic Injection of PFFs

For stereotaxic injections, the PFFs were diluted in sterile PBS and fragmented using a Bioruptor bath sonicator (Diagenode, Denville, NJ). Sonication was performed at high power for 10 cycles (30s on, 30s off, at 10 °C). Mice were anesthetized with ketamine hydrochloride (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). For each animal, PFFs were stereotaxically targeted into the ventral striatum (AP: +0.2 mm Bregma, lateral: 2.0 mm from midline, depth: 3.6 mm beneath the dura), dorsal striatum (AP: +0.2 mm, lateral: 2.0 mm, depth: 2.6 mm), and overlaying cortex (AP: +0.2 mm, lateral: 2.0 mm, depth: 0.8 mm). Injections were made through a single needle tract using 10 μL syringes (Hamilton, NV) at a rate of 0.1 μL per min (2.5 μL total per site) with the needle in place for ≥5 min at each target. Animals were monitored regularly following recovery from surgery. Mice were sacrificed at 90 days post injection by overdose with ketamine/xylazine. For biochemical studies, dorsal striatum and ventral midbrain from ipsilateral and contralateral sides were dissected and stored at −80 °C until used. For histological studies, the brain and spinal cord were removed after transcardial perfusion with PBS and underwent overnight post-fixation in either neutral buffered formalin (Fisher Scientific) or 70% ethanol (in 150 mM NaCl, pH 7.4), before being processed and embedded in paraffin.

2.3. Immunohistochemistry and Neuron Counting for Mouse Brain

Immunohistochemistry for α-synuclein phosphorylated at Ser-129 and tyrosine hydroxylase (TH) were performed on 6 μm thick coronal sections as previously described [18]. Digitized images of stained sections were acquired using a Perkin Elmer Lamina scanner at 20× magnification. Midbrain dopaminergic neurons belonging to the substantia nigra pars compacta and the ventral tegmental area were quantified from TH-immunostained coronal sections spanning the entire extent of the midbrain (every 9th section). Only intact neurons with visible nuclei and TH positive staining were included in the counting based on established criteria [26]. Statistical analysis between groups was compared using unpaired *t*-test.

2.4. Sample Preparation and LC-MS/MS Analysis

For each mouse injected, the midbrain and striatum of the injected and non-injected sides were individual dissected and kept separate. Two midbrain and striatum regions of the injected hemisphere were combined to generate one biological sample for the proteomic analysis. The same approach was employed for the non-injected side. Four biological samples for wild type and three for *Snca*$^{-/-}$ for each injected and non-injected side were analyzed through the proteomic workflow. Homogenates were prepared as described previously [27]. Briefly, brains were homogenized with a tissue grinder in cold urea buffer: 8 M urea, 75 mM NaCl, 50 mM Tris HCl pH 8.0, 1 mM EDTA, 2 μg/mL aprotinin (Sigma, A6103), 10 μg/mL leupeptin (Roche, 11017101001), 1 mM PMSF (Sigma, 78830), 10 mM NaF, 5 mM sodium butyrate, 5 mM iodoacetamide (Sigma, A3221), Phosphatase Inhibitor Cocktail 2 (1:100, Sigma, P5726), and Phosphatase Inhibitor Cocktail 3 (1:100, Sigma, P0044). Following 10 min centrifugation at 20,000*g*, protein concentration was determined by a BCA assay (Thermo, 23235). The supernatant was then combined with ^{13}C-labeled brain lysates in a 1:1 ratio (5 μg). Samples were reduced for 45 min with 5 mM dithiothreitol followed by alkylation with 20 mM iodoacetamide for 45 min. Samples were then diluted 1:4 with 50 mM Tris HCl pH 8.0 (to reduce urea concentration to 2 M), then digested overnight with trypsin (Promega, V5111) at 37 °C overnight. 1% formic acid was added to the digests to remove urea by pelleting. The tryptic peptides were desalted by ultraMicro-Spin Vydac C18 column (Nestgroup, Inc., SUMSS18V). After peptide separation by high-pH reverse phase chromatography, 95% of peptides were combined in a concatenated pattern into 12 fractions for phosphoproteomic analysis. Lyophilized phosphopeptides fractions were re-suspended in 50% acetonitrile/0.1% trifluoroacetic acid (TFA) and then diluted 1:1 with 100% acetonitrile/0.1% TFA. These samples were then enriched for phosphorylation by incubation with 10 μL immobilized metal affinity chromatography (IMAC) for 30 min. Enriched IMAC beads were the loaded onto C18 silica-packed stage tips washed twice with 50 μL of 80% acetonitrile/0.1% TFA and 100 μL of 1% formic acid. Phosphopeptides were then eluted from IMAC