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Convergence of Heat Shock Protein 90 with Ubiquitin in Filamentous α-Synuclein Inclusions of α-Synucleinopathies

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Heat shock proteins (Hsps) facilitate refolding of denatured polypeptides, but there is limited understanding about their roles in neurodegenerative diseases characterized by misfolded proteins. Because Parkinson's disease (PD), dementia with Lewy bodies, and multiple system atrophy are α-synucleinopathies characterized by filamentous α-synuclein (α-syn) inclusions, we assessed which Hsps might be implicated in these disorders by examining human brain samples, transgenic mouse models, and cell culture systems. Light and electron microscopic multiple-label immunohistochemistry showed Hsp90 was the predominant Hsp examined that co-localized with α-syn in Lewy bodies, Lewy neurites, and glial cell inclusions and that Hsp90 co-localized with α-syn filaments of Lewy bodies in PD. Hsp90 levels were most predominantly increased in PD brains, which correlated with increased levels of insoluble α-syn. These alterations in Hsp90 were recapitulated in a transgenic mouse model of PD-like α-syn pathologies. Cell culture studies also revealed that α-syn co-immunoprecipitated preferentially with Hsp90 and Hsc70 relative to other Hsps, and exposure of cells to proteasome inhibitors resulted in increased levels of Hsp90. These data implicate predominantly Hsp90 in the formation of α-syn inclusions in PD and related α-synucleinopathies. ***(Am J Pathol 2006, 168:947–961; DOI: 10.2353/ajpath.2006.050770)***

α-Synuclein (α-syn) is a constituent of filamentous Lewy bodies (LBs) and Lewy neurites (LNs) in Parkinson's disease (PD).[1–3] Three missense mutations in the α-syn gene as well as gene duplications/triplications have been reported in familial PD with or without dementia.[4–7] In addition, LBs and LNs are the defining lesions of dementia with LBs (DLB) and the LB variant of Alzheimer's disease (LBVAD).[8] Glial cytoplasmic inclusions (GCIs) consisting of filamentous α-syn in oligodendrocytes are hallmarks of multiple system atrophy (MSA), a neurodegenerative movement disorder.[9] Although α-syn is ubiquitinated in α-syn inclusions, the significance of this is not clear because native α-syn fibrillizes *in vitro* and other abnormal modifications of α-syn have been implicated in the formation of α-syn inclusions.[10–15] However, overexpression of mutant or wild-type α-syn in transgenic (Tg) animals has yielded models of neurodegenerative α-synucleinopathies with inclusions formed by aggregated α-syn filaments.[16–19]

Accumulation of misfolded proteins due to a variety of stress situations leads to up-regulation of heat shock proteins (Hsps) to facilitate refolding and prevent aggregation of misfolded proteins.[20,21] Hsps may help ubiquitinate and target nonrepairable proteins to the proteasome.[22] Although proteasome impairments are linked to the formation of inclusions in neurodegenerative disorders, including Alzheimer's disease (AD) and

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Table 1. List of Antibodies Used in This Study

Antibody	Code	Host	Dilution	Source
αB-Crystallin	1B6.1-3G4	Mouse	2.5 k	StressGen (Victoria, BC, Canada)
Hsp27	a28BG	Rabbit	500	W. Welsh
Hsp27	G3.1	Mouse	1 k	W. Welsh
Hsp27	D5	Mouse	500	W. Welsh
Hsp40	Hsp40	Rabbit	1 k	W. Welsh
Hsp60	LK-1	Mouse	500	StressGen
Hsc70	IB5	Rat	1 k	W. Welsh
Hsc70	Hsp72	Rabbit	800	W. Welsh
Hsp70	C92 F3-4	Mouse	1 k	W. Welsh
Hsp90	AC88	Mouse	200	StressGen
Hsp90	9D2	Rat	4 k	W. Welsh
α-Syn	syn303	Mouse	5 k	CNDR
α-Syn	SNL4	Rabbit	200	CNDR
α-Syn	LB509	Mouse	1 k	T. Iwatsubo (University of Tokyo, Tokyo, Japan)
α-Syn	syn310	Mouse	1 k	CNDR
Tau	17024	Rabbit	1 k	CNDR
Tau	N-tau	Rabbit	1 k	CNDR
Tau	PHF-1	Mouse	1 k	P. Davies (Albert Einstein College of Medicine, Bronx, NY)
Ubiquitin	1510	Mouse	10 k	Chemicon
Ubiquitin	P4G7-H11	Mouse	1 k	StressGen
Tubulin	α-Tubulin	Mouse	1 k	Sigma
CHIP	AQP1	Rabbit	500	Chemicon (Temecula, CA)
MBP	MBP	Rabbit	1 k	A. McMorris (Wister Institute, Philadelphia, PA)

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PD,[23,24] Hsps can also be induced by proteasome inhibitors.[25–27] Various Hsps have been found in cytoplasmic inclusions of neurons and glia in diverse neurodegenerative diseases.[28,29] Hsp90 is an abundant molecular chaperone that prevents protein aggregation and increases Hsp expression.[30] Although it has an ATPase domain, Hsp90 can act independently of ATP and can prevent protein aggregation alone or with other chaperones, such as Hsp70.[30] Although several studies suggest Hsp70 plays a mechanistic role in α-synucleinopathies,[18,31–33] numerous other Hsps are expressed in brain, and it is not clear if Hsp70 alone versus multiple brain Hsps are involved in α-synucleinopathies or if Hsps contribute to mechanisms of disease in other neurodegenerative disorders. We addressed these questions here by systematically screening the brains of patients with α-synucleinopathies and other disorders as well as animal models of a PD-like α-synucleinopathy for pathological alterations of multiple brain Hsps. Data from these studies plus other data from cell culture systems implicate Hsp90 as the predominant Hsp implicated in α-syn pathologies in diverse α-synucleinopathies but not in other neurodegenerative disorders.

Materials and Methods

Antibodies

The panel of antibodies to Hsp, α-syn, tau, and ubiquitin used in this study, including their respective dilutions and origins, are summarized in Table 1.

Human Brain Tissue and Patient Demographics

Brain tissue was obtained from the Center for Neurodegenerative Disease Research and the AD Center Core at the University of Pennsylvania School of Medicine. Established diagnostic criteria were used to assign brains to normal or disease groups as described.[34–40] Demographic information for the patients analyzed is presented in Table 2. Age, postmortem interval, or gender did not show a noticeable effect on the results of the following studies.

Table 2. Demographics of Patients Analyzed in This Study

Neuropathological diagnosis	Number of cases analyzed	Age, years (range)	Gender (M:F)	PMI, hours (range)
Normal	6	69 (43–92)	2:4	11 (3–31)
PD	9	69 (75–83)	3:6	15 (4–44)
DLB	11	75 (58–90)	5:6	8 (2–16)
ADLBV	8	75 (62–81)	4:4	13 (6–20)
MSA	10	64.5 (43–79)	6:4	17 (7–43)
AD*	7	88 (67–96)	4:3	11 (4–22)
FTD-MND	6	59 (48–77)	3:3	10 (5–16)

M, male; F, female; PMI, post-mortem interval.
*Braak and Braak stage V/VI.[71]

α-Syn Tg Mice

Mice from stable Tg mouse lines carrying the A53T human α-syn mutation (line M83, 3 and 9 months old) and wild-type human α-syn (line M7, 9 months old) as well as non-Tg mice (C57BL/C3H) (9 months old) were studied using immunohistochemical (IHC) methods previously reported in the characterization of these Tg mice.[19]

IHC Analyses

Preliminary studies were performed with antibodies against various Hsps and the carboxyl terminus of Hsp70 interacting protein (CHIP) to optimize IHC analyses, including examination of various fixatives (10% formalin or 70% ethanol) and antigen retrieval pretreatments (with or without microwaving, boiling, or 88% formic acid) on 6-μm-thick paraffin sections or fresh frozen brain and cryosectioning (10 μm thick) followed by 10% formalin fixation as described.[41–43] As summarized in the Results, screening of multiple antibodies to six different Hsps showed that Hsp90 was most prominent in filamentous α-syn inclusions, and paraffin-embedded sections cut from 70% ethanol-fixed tissues were used for IHC without antigen retrieval, except as noted. IHC was performed as described using the avidin-biotin complex (ABC) method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine (DAB) as chromogen.[44,45] Double-labeling IHC with antibodies specific for Hsp90 and α-syn (SLN4, syn303) was conducted using either the ABC method with DAB followed by the β-galactosidase-based ABC method (Amersham Biosciences Corp., Buckinghamshire, UK) with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) visualization[46] or two-color fluorescent IHC (FIHC) and secondary antibodies conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR) and Texas Red (Jackson ImmunoResearch Laboratory, West Grove, PA) or Alexa Fluor 594 (Molecular Probes) as described.[45] Triple-labeling FIHC studies were performed by co-incubating sections with antibodies specific for Hsp90, α-syn, and tau or ubiquitin, raised in different species, followed by visualizing bound antibodies with secondary antibodies conjugated with Alexa Fluor 488, Texas Red, or AMCA (Vector Laboratories) as described.[45,47] Autofluorescence was blocked using published methods.[48,49] These sections were coverslipped using Vector Shield mounting medium (Vector Laboratories) with (for double stain) or without (for triple stain) 4,6-diamino-2-phenylindole (DAPI) (1 μg/ml). Negative and positive controls for IHC and FIHC studies were performed as reported,[44,47] and sections were analyzed with an Olympus BX51 microscope (Tokyo, Japan) equipped with bright-field and fluorescence light sources. Images were captured using a ProGres C14 digital camera (Jenoptik AG, Jena, Germany).

To estimate the relationship between Hsp90 and tau or α-syn lesions, as well as α-syn, Hsp90, and ubiquitin, semiquantitative analyses of double- and triple-labeled FIHC sections were conducted using a combination of different antibodies to these proteins applied to sections of the amygdala from PD, DLB, LBVAD, AD, and normal brains ($n = 6$), as well as the pons from MSA and normal brains ($n = 6$). Briefly, micrographs of five fields from the triple-label FIHC sections were captured (with ×20 objective lens) within the anatomical area of interest. Images were opened with ImagePro^{plus} (Media Cybernetics, Silver Spring, MD), thresholds were established, manual editing was performed to eliminate artifacts, and positively stained profiles were counted. After importing images into Photoshop (Adobe System, San Jose, CA), sets of images labeled by Alexa Fluor488, Texas Red, and/or AMCA were overlaid, and double- as well as triple-labeled objects were counted and statistically analyzed. Positively labeled objects were included regardless of shape, and profiles smaller than the average diameter of axons (3 pixels in ImagePro^{plus}) were not included in this analysis.

Immunoelectron Microscopy

For immunoelectron microscopy, samples of midbrain were excised from the brains of three patients with PD (with a 3- to 4-hour postmortem interval), fixed in 4% paraformaldehyde and 0.1% glutaraldehyde overnight, and cut into 50-μm-thick sections using a Vibratome 3000 microtome (Vibratome, St. Louis, MO). Sections including LBs and LBs visualized by eosin staining were embedded in LR White. Ultrathin sections were used for single- or double-immunoelectron microscopy studies as described with minor modifications.[50] Ultrathin sections (70 nm) were cut and double immunolabeled using mouse anti-α-syn monoclonal antibody (mAb) syn303 and rat anti-Hsp90 mAb 9D2 followed by anti-mouse IgG and anti-rat IgG conjugated with 10-nm protein A gold and 18-nm protein L gold (Rockland Immunochemical, Gilbertsville, PA), respectively. Chemicals for immunoelectron microscopy were purchased from Electron Microscopic Sciences (Fort Washington, PA).

Sequential Biochemical Fractionation and Western Blot Analyses

For sequential biochemical fractionation and Western blot studies, samples of frozen cingulate cortex from PD, DLB, LBVAD, and normal control as well as pons from MSA and normal control ($n = 6$) were examined using previously described methods.[19] Brain tissue was homogenized in 3 ml/g of high-salt (HS) buffer [50 mmol/L Tris, pH 7.5, 750 mmol/L NaCl, 5 mmol/L ethylenediamine tetraacetic acid (EDTA), and protease inhibitor cocktail]. The samples were centrifuged at 100,000 × *g* for 30 minutes. Subsequently, the pellets were homogenized with 3 ml/g of HS buffer containing 1% Triton X-100 (HS/T fraction) and centrifuged at 100,000 × *g* for 30 minutes. The pellets were homogenized in 500 μl of HS buffer containing 1 mol/L sucrose and centrifuged at 100,000 × *g* for 30 minutes. Floating myelin was discarded, and pellets were extracted with 2 ml/g of radioimmunoprecipitation assay (RIPA) buffer [50 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Nonidet P-40, 0.5%

sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)] and centrifuged at 100,000 × *g* for 30 minutes. The pellets were homogenized in SDS buffer (10 mmol/L Tris, pH 6.8, 1 mmol/L EDTA, 40 mmol/L dithiothreitol, 1% SDS, 10% sucrose) and centrifuged at 100,000 × *g* for 30 minutes. The pellet was sonicated in 1 ml/g 70% formic acid (FA). FA was removed by lyophilization. SDS-sample buffer (10 mmol/L Tris, pH 6.8, 1 mmol/L EDTA, 40 mmol/L dithiothreitol, 1% SDS, 10% sucrose) was added to HS, HS/T, RIPA, and FA fractions and samples were heated at 100°C for 5 minutes. The same amount of each fraction was loaded on separate lanes of 15% polyacrylamide gels and subjected to Western blot analysis as described previously.[19,51]

Cell Culture

Cell culture media was purchased from Invitrogen (Grand Island, NY). Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132) and lactacystin were purchased from Calbiochem (Bad Soden, Germany). Cells were maintained at 37°C, 10% CO_2. To study the effects of proteasome inhibition on cultured cells, we used rodent OLN-93 cells, a permanent oligodendroglial cell line.[52] These cells were stably transfected with cDNAs to express the longest human tau isoform (tau40)[53] and α-syn, respectively. OLN-tau40-α-syn cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Primary cultures of rat brain oligodendrocytes were examined. To do this, primary cultures of glial cells were prepared from the brains of 1- to 2-day-old Wistar rats, and oligodendrocytes were prepared from the flasks after 6 to 8 days as described.[54] Precursor cells were replated on poly-L-lysine-coated culture dishes (2.7×10^6cells/10 cm dish) and kept for 5 to 7 days in serum-free Dulbecco's modified Eagle's medium to which insulin (5 μg/ml), transferrin (5 μg/ml), and sodium selenite (5 ng/ml) (Roche Diagnostics, Mannheim, Germany) were added. These cultures contain a highly enriched population of differentiated oligodendrocytes with a mature morphology.

For proteasome inhibition studies, cells were treated with MG-132 as indicated, and cellular monolayers of control and treated cells were washed with phosphate-buffered saline once, scraped into sample buffer, and heated for 10 minutes. Protein contents in the samples were determined as described.[55] Total cellular extracts were separated by SDS-polyacrylamide gel electrophoresis followed by Western blot analysis as described above. The following antibodies were used for these studies: anti-α-tubulin (1:1000) (Sigma, Taufkirchen, Germany), anti-ubiquitin (P4G7-H11, 1:1000), and anti-Hsp90 (AC88; 1:1000) (StressGen, Victoria, BC, Canada) mouse mAb, and anti-myelin basic protein rabbit polyclonal antibody (1:1000) (gift from Dr. A. McMorris, Wistar Institute, Philadelphia, PA).

RNA Extraction and Reverse Transcription

RNA from oligodendrocytes (2.7×10^6 cells) was isolated with the RNeasy kit (Qiagen, Hilden, Germany) as described by the manufacturer for animal cells. One μg of RNA was used for reverse transcription in a final volume of 20 μl and polymerase chain reactions were performed in a Biometra Thermocycler (Göttingen, Germany) as previously described.[56] Reaction products were analyzed on agarose gels and visualized by ethidium bromide staining. Primers were synthesized by Gibco-BRL Life Technologies (Karlsruhe, Germany). Primers were designed using PrimerSelect software (DNASTAR, Madison, WI).

For the analysis of Hsp90 the following oligonucleotides were used: 5′-GAT TGA CAT CAT CCC CAA CC-3′ and 5′-CTT CAT CAG ATC CCA CAT CC-3′. Control experiments were performed with the following primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5′-CCCACGGCAAGTTCAACGGCA-3′ (nucleotides 220 to 240) and 5′-TGGCAGGTTTCTCCAGGCGGC-3′ (nucleotides 805 to 825).[57]

Co-Immunoprecipitation

OLN cells stably expressing tau and α-syn were homogenized in 1 ml of RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris, pH 8.0, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) with protease inhibitors. The homogenates were spun at 3000 rpm for 5 minutes to yield lysate. The lysate was precleared with protein A/G agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and subsequently used for immunoprecipitation reactions. Sufficient quantities of either anti-α-syn (syn310), anti-Hsp90 (AC88), anti-Hsp70 (C92 F3–4), anti-Hsc70 (IB5), or anti-Hsp40 (rabbit) antibody or no antibody was added to the precleared lysate in an individual test tube so as to completely immunoprecipitate the antigens in the lysate. The antibody-protein complex was precipitated with protein A/G agarose. The beads were washed several times with RIPA buffer, once with 50 mmol/L Tris, pH 6.8, and resuspended in 2× SDS loading buffer. Equivalent volumes of immunoprecipitates and supernatants from immunoprecipitates were loaded in each lane and subsequently analyzed by Western blotting as described.

Statistical Analyses

Data for the number of inclusions and double-labeled elements from the studies described above were expressed as mean ± SEM. The ratio of Hsp90, tau, and ubiquitin immunoreactivity (IR) over α-syn or tau IR values was assessed by analysis of variance and subsequently by unpaired, two-tailed Student's *t*-test, taking into consideration disease type and protein type. Significance was set at $P < 0.05$.

Results

IHC Screening of Hsps in α-Synucleinopathies

The extent to which several major Hsps (αB-crystallin, Hsp27, Hsp40, Hsp60, Hsp70, Hsc70, and Hsp90) co-localize in LBs and GCIs among various α-syn inclusions in human disease brains was assessed by double-label FIHC screening with the combination of antibodies listed in Table 1. Antibodies to Hsp27 (~70%), Hsp40 (~60%), Hsc70 (~75%), and Hsp90 (~95%) labeled large subsets of LBs, whereas αB-crystallin, Hsp60, or Hsp70 were nearly undetectable in these inclusions (~5%) (Figure 1, a–g). GCIs were consistently detected by antibodies to αB-crystallin (>90%) and Hsp90 (>90%) but rarely by Hsp27 (~10%), Hsp40 (~10%), or Hsp70 (<5%) (Figure 1, h–n). We used confocal microscopy to confirm co-localization of these proteins in studies preliminary to the more detailed analyses reported in our manuscript. In view of this, and because epifluorescence microscopy is much more practical and feasible for large scale quantitative analysis as conducted here, we do not describe the data from these preliminary confocal analyses in the present study.

Hsp90 Immunoreactivity in Human Brain

Specificity of the Hsp90 mAbs used was confirmed as shown in Figure 1, where rat 9D2 recognized a discrete band at 84 kd in HS extracts of human, but not mouse, brain extracts (Figure 1o). Mouse AC88 labeled a distinct 84-kd band in both mouse and human brain HS homogenates (Figure 1o).

In normal human brain, both anti-Hsp90 antibodies stained neuronal perikarya and proximal dendrites throughout all brain regions examined and, to a lesser extent, glial cells (Figure 1p). Brain sections from patients with PD, DLB, LBVAD, MSA, and frontotemporal dementia with motor neuron disease type (FTD-MND) showed ubiquitin inclusions with a modest basal staining pattern similar to normal control brains (Figure 1u). However, in contrast to controls, intense Hsp90 immunoreactivity (iHsp90) IR was observed in LBs, LNs, spheroids of the PD, DLB, and LBVAD brain sections (Figure 1, q and r), and in the GCIs of MSA (Figure 1s). In sharp contrast, neurofibrillary tangles in AD and DLB brain sections showed little or no Hsp90 IR (Figure 1, r and t) compared with prominent tau-positive staining of neurofibrillary tangles by AT8 in the identical area in the adjacent section of CA1 region of AD brain (Figure 1t, inset). Occasionally increased Hsp90 IR was noted in nontangle-bearing neurons (data not shown). The composition of the diagnostic inclusions in FTD-MND is not well characterized, but they are identified by ubiquitin IHC and showed modest Hsp90 IR (Figure 1u and inset therein).

In double-label IHC studies using horseradish peroxidase-DAB (for Hsp90) and β-galactosidase-X-Gal (for α-syn), Hsp90 IR clearly co-localized with α-syn in LBs and LNs of PD, DLB, and LBVAD brains (Figure 1v) and in GCIs and threads in MSA brains (Figure 1w). This result was further confirmed using double-label FIHC methods, which demonstrated iHsp90 IR in α-syn IR LBs and LNs (Figure 2, a–c) as well as in GCIs (Figure 2, d–f). Because variable amounts of tau pathologies were noted in the brains of patients with different neurodegenerative diseases, including tauopathies and α-synucleinopathies, as well as in normal control, and tau may co-exist in filamentous α-syn lesions,[51,58] triple-label FIHC was performed on sections of control and disease brains using antibodies raised in different species, ie, rabbit anti-tau (17024), mouse anti-α-syn, and rat anti-Hsp90 (9D2), or rabbit anti-tau (N-tau), mouse anti-α-syn, and rat anti-Hsp90 (9D2). Figure 2 illustrates representative data from these studies, including the presence of tau-positive neurites, some of which were iHsp90-positive. However, there was greater co-localization of iHsp90 IR and α-syn than iHsp90 IR and tau in neuronal or glial inclusions containing either tau or α-syn (Figure 2, g–j). Localization of CHIP in α-syn inclusion was also examined, but no CHIP-positive staining was found on LBs, LNs, or GCI (Figure 1x).

Semiquantitative analyses were conducted on sections of amygdala, which commonly harbors inclusions in diverse neurodegenerative diseases. These studies confirmed that 1) the occurrence of both tau and α-syn pathologies in amygdala of AD and α-synucleinopathies; 2) the abundance of tau-positive thread pathology in α-synucleinopathies; and 3) co-localization of iHsp90 IR with α-syn IR in the inclusions of α-synucleinopathies, including LBs, LNs, and GCIs, significantly more often than with tau IR (Figure 2, k and l).

Hsp90 and Ubiquitin in α-Syn Pathologies

Because ubiquitinated α-syn in α-syn inclusions suggests a functional relationship between Hsp90 and ubiquitin, we analyzed the distribution of Hsp90 and ubiquitin in α-syn inclusions, including cell body and thread pathologies, in the amygdala of PD, DLB, LBVAD brains, and the pons from MSA brains using triple-label FIHC. These studies showed that not every α-syn IR element was Hsp90 IR or ubiquitin IR and that Hsp90 IR was more common in ubiquitin-positive, as compared to ubiquitin-negative, α-syn lesions in all α-synucleinopathy brains examined (Figure 3, a–h). These observations were confirmed using semiquantitative analysis (Figure 3, i and j). In follow-up semiquantitative analyses using double-label FIHC and DAPI staining, the ratio of co-localization of iHsp90 IR or ubiquitin IR with α-syn IR was ~90% in DLB and MSA (Figure 3, i and j). Thus, both Hsp90 and ubiquitin may be sequestered into α-syn inclusions by linked mechanisms because they co-occur in the same α-syn lesions more often than separately.

Western Blot Analysis of Human α-Synucleinopathy Brains

α-Syn, Hsp90, Hsp70, Hsc70, Hsp40, Hsp27, αB-crystallin, and ubiquitin protein levels were assessed and compared biochemically using sequential fractionation and Western blot analysis. In HS fractions of cingulate cortex

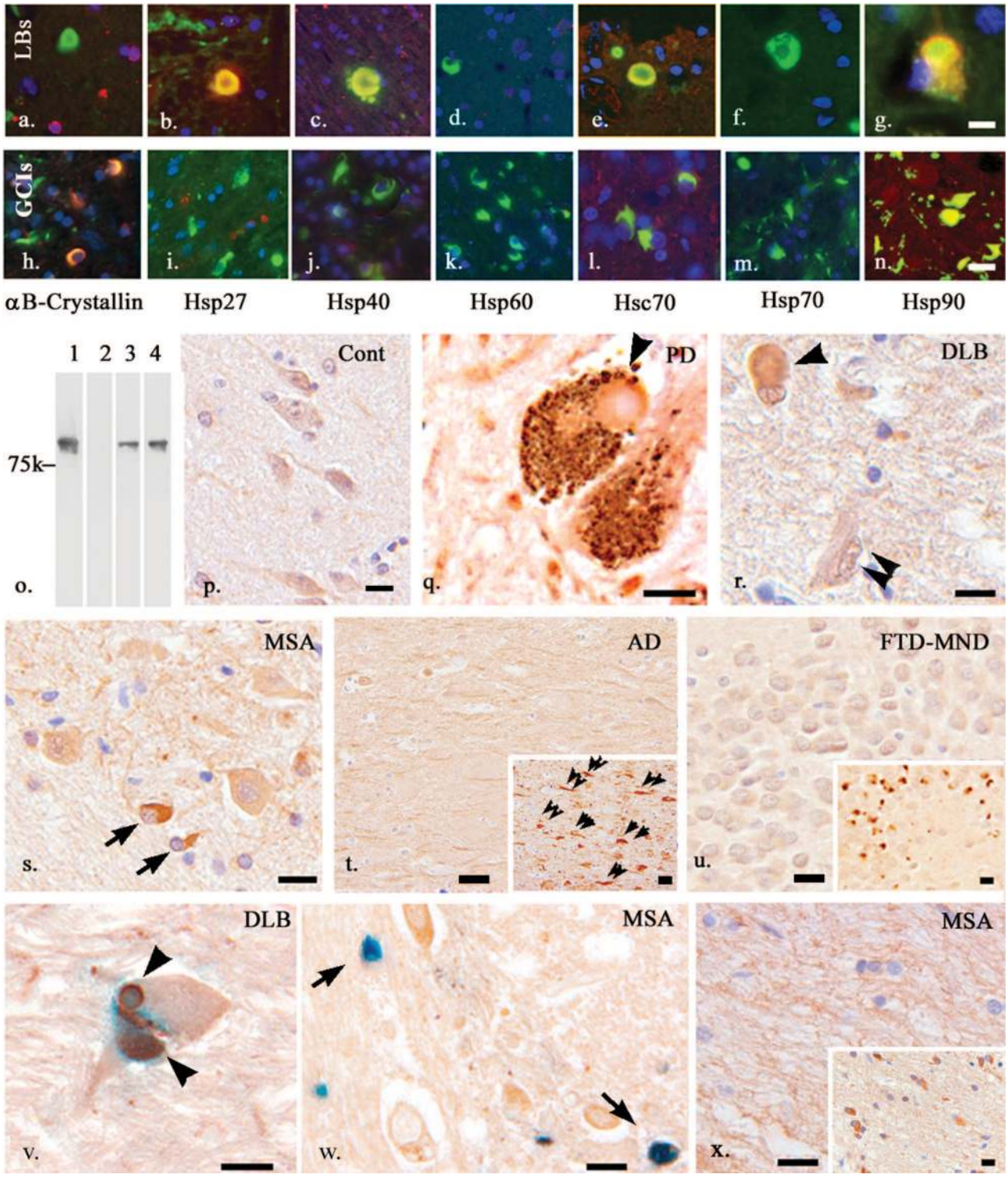

Figure 1. Screening of Hsps using double-label FIHC with different anti-Hsp and anti-α-syn antibodies visualized by Alexa Fluor 594 or Texas Red and Alexa Fluor 488, respectively, shows co-localization of a subset of Hsps examined in LBs (**a–g**) and GCIs (**h–n**). Yellow color represents co-localization of Hsp and α-syn. Hsp27 (**b**), Hsp40 (**c**), Hsc70 (**e**), and Hsp90 (**g**) are located in LBs, whereas αB-crystallin (**h**) and Hsp90 (**n**) are located in GCIs and threads. Specificity of Hsp90 antibodies (**o**), 9D2 (**lanes 1** and **2**), and AC88 (**lanes 3** and **4**) is shown in HS fraction of human (**lanes 1** and **3**) and mouse (**lanes 2** and **4**) brain homogenates. Rat 9D2 does not recognize mouse Hsp90 (**lane 2**). Photomicrographs in **p–x** show modest Hsp90 IR in neurons of normal brain (**p**), which also is seen in α-synucleinopathy brains, in addition to more intense Hsp90 IR in LBs (**arrowhead**) of the PD SN (**q**), DLB amygdala (**r**), and GCIs (**arrow**) in the MSA pons (**s**). Little or no iHsp90 IR is seen in neurofibrillary tangles of AD (**t**, **double arrowheads**) and DLB (**r**). **t:** IHC profile with an antibody against Hsp90 (9D2) or tau (AT8) (**inset**) from adjacent sections in the CA1 region of AD hippocampus are presented for comparison. It is apparent that Hsp90 IR is reduced in neurofibrillary tangles. **u:** Modest Hsp90 IR is noted in ubiquitin inclusions in the hippocampus of the FTD-MND brain. **Inset** shows ubiquitin IR in inclusions on the adjacent section. **v** and **w** show double-label IHC with horseradish peroxidase-DAB and β-galactosidase-X-gal to illustrate co-localization of iHsp90 IR (brown) with α-syn IR (blue) in LBs (**arrowhead**) in the midbrain of PD (**v**) as well as GCIs (**arrow**) in the pons of MSA (**w**). **x:** No CHIP IR was found on GCI in the pons of MSA, whereas the adjacent section indicates descent α-syn IR inclusions (**inset**). Scale bars, 10 μm.

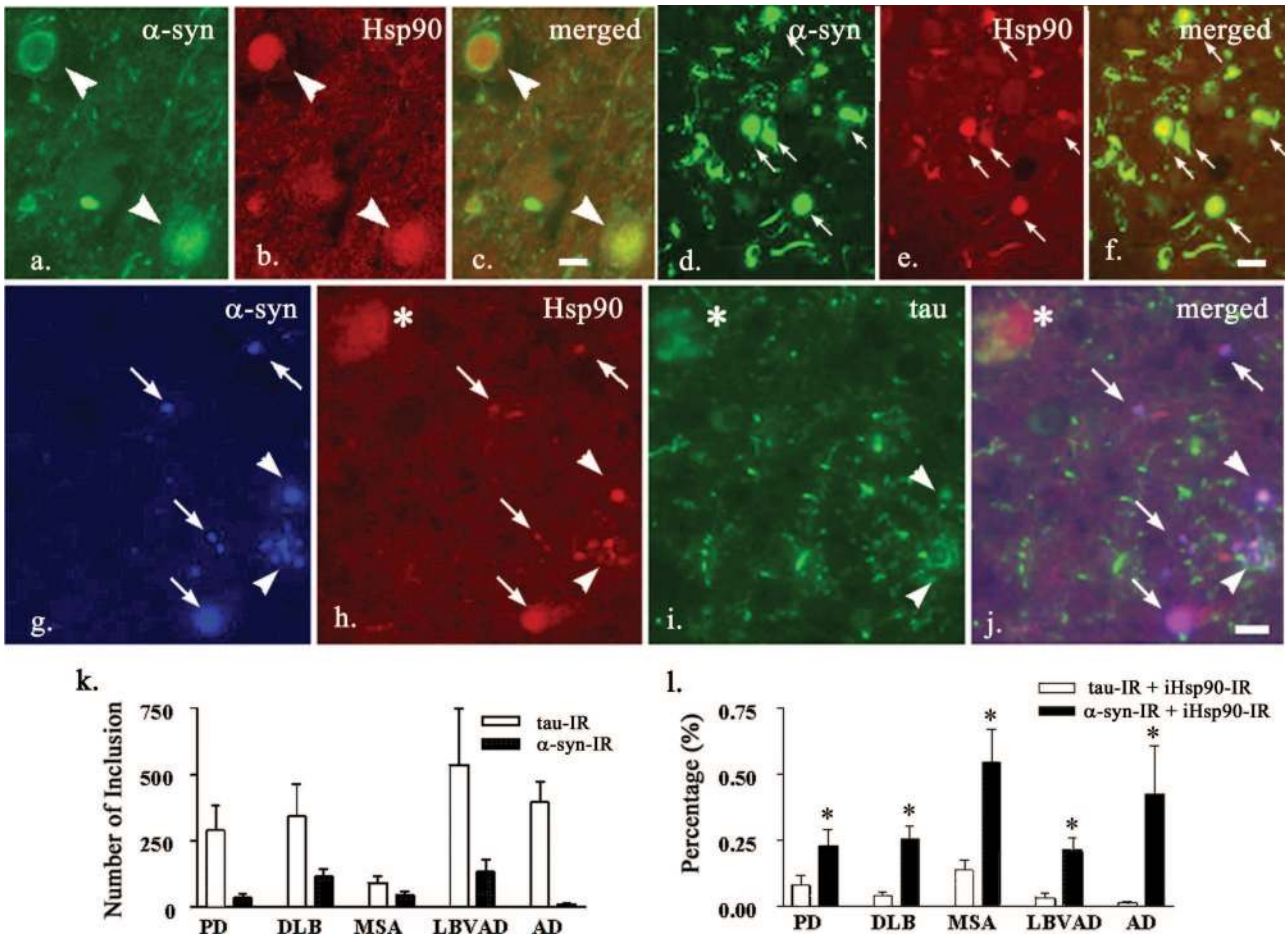

Figure 2. Double-label FIHC confirms Hsp90 IR (Texas Red) co-localizes with α-syn IR (Alexa Fluor 488) in LBs of PD amygdala (**arrowhead** in **a–c**) and GCIs in the MSA pons (**d–f**). **g–j:** Representative images from triple-label FIHC of DLB amygdala shows differential co-localization of Hsp90 with α-syn (**arrow**) rather than tau-positive inclusions (**arrowhead**) in these preparations. Even when tau IR is located in iHsp90 IR cells, the subcellular localization of these proteins is normally discordant (**asterisk**). **k:** Semiquantitative analysis reveals the number of iHsp90 IR with α-syn- or tau IR lesions including intracytoplasmic inclusions, dystrophic neurites, and spheroids. **l:** iHsp90 co-localizes with α-syn to a significantly greater extent ($P < 0.01$) than tau in disease lesions. **Asterisks** indicate statistically significant difference. $P < 0.05$. Scale bars, 10 μm (**a–j**).

from PD, DLB brains, and pons of MSA brains, similar amounts of monomeric α-syn were noted, whereas high-molecular weight species of α-syn were visible in the FA-insoluble fractions from PD, DLB, and MSA cases in association with moderately elevated levels of Hsp90 protein in these same fractions in a consistent manner (Figure 4). Also there was a slight increase in Hsc70 in FA fractions of PD, DLB, and MSA, whereas Hsp70 levels did not differ in disease versus normal brains throughout the fractions, and, in addition, Hsp70 was not detectable in FA fraction (Figure 4). Moderate accumulation of Hsp40 and Hsp27 relative to normal brains also was noted in FA fractions of PD and DLB brains, and slight accumulation of αB-crystallin was observed in the FA fraction of MSA brains (Figure 4). Polyubiquitinated protein species were detectable only in FA fractions, and the levels of these proteins were greater in α-synucleinopathy than normal brains (Figure 4). These finding are consistent with the distribution of these proteins in α-syn inclusions of different disease brains as described above.

Up-Regulation of Hsp90 and Ubiquitin in Oligodendroglial and Neuronal Cell Cultures

Because impairments in proteasome activity have been implicated in α-synucleinopathies,[59] we examined if proteasomal inhibition might be causally related to the sequestration of Hsp90 in LBs and GCIs. To address this question, rat primary oligodendrocytes were prepared and treated with MG-132 or lactacystin. MG-132 induced the accumulation of ubiquitinated proteins and Hsp90, which was maximal after 18 hours of treatment (Figure 5Aa), but there was no change in levels of tubulin or myelin basic protein (Figure 5Aa). This effect was observable after treatment with MG-132 at concentrations as low as 0.1 μmol/L (Figure 5Ab). Similarly, treatment with lactacystin (10 μmol/L, 18 hours), a more specific proteasome inhibitor, caused the induction of ubiquitinated proteins and Hsp90 (Figure 5Ab). MG132 or lactacystin did not cause apparent alterations of α-syn protein levels or noticeable oligomerization of the molecule under the spe-

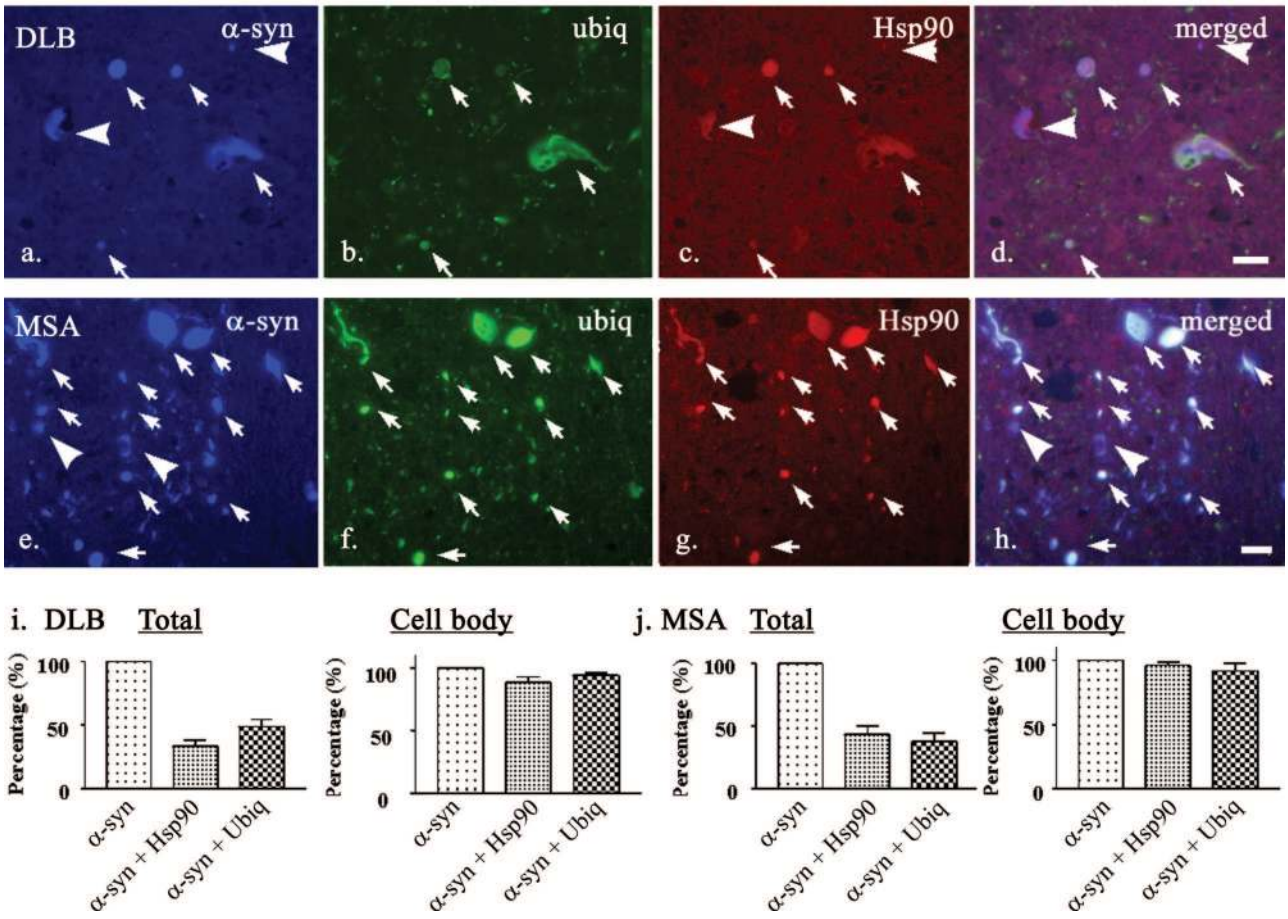

Figure 3. Triple-label FIHC micrographs of iHsp90 (Texas Red), ubiquitin (Alexa Fluor 488), and α-syn (AMCA) of DLB amygdala (**a–d**) and MSA pons (**e–h**) indicate that iHsp90 co-localizes preferentially with ubiquitinated α-syn lesions (**arrow**), whereas a subset of α-syn-positive profiles are ubiquitin-negative (**arrowhead**). **i** and **j:** Data from semiquantitative analyses of triple-label FIHC studies of amygdala from DLB brain (n = 6 in **i**) and pons of MSA brains (n = 6 in **j**) showing that a similar percentage (~30 to 45%) of the total number α-syn lesions (neurites plus perikaryal inclusions) are iHsp90− (α-syn + Hsp90) and ubiquitin IR (α-syn + Ubiq), whereas >90% of perikaryal α-syn inclusion lesions are iHsp90− (α-syn + Hsp90) and ubiquitin IR (α-syn + Ubiq). There were no significant differences between the number of Hsp90-positive and ubiquitin-positive α-syn inclusions. Scale bars, 10 μm (**a–h**).

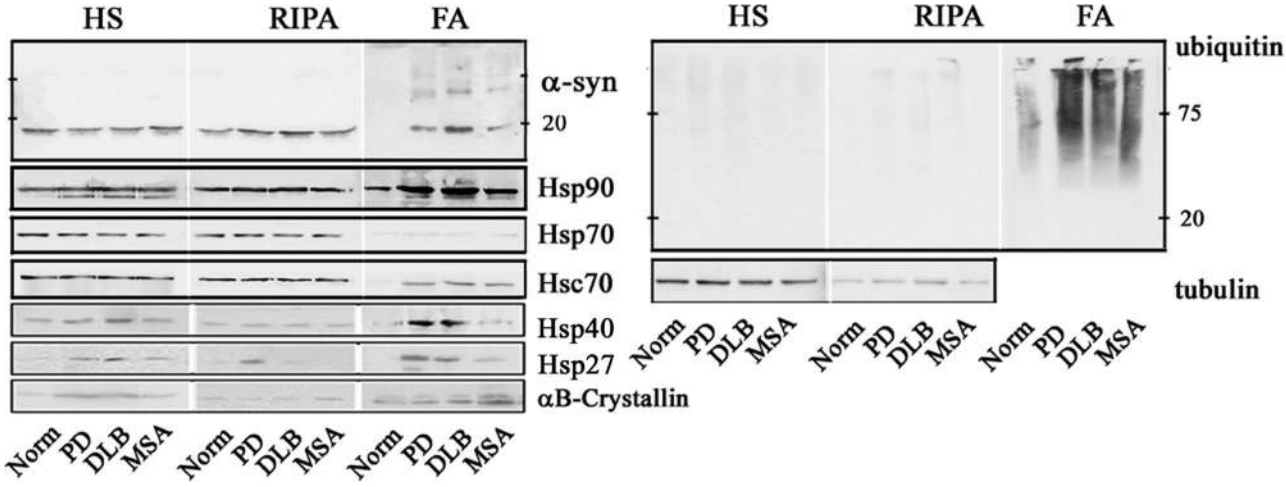

Figure 4. Representative images from Western blot analyses (WB) of HS, RIPA, and FA samples of cingulate cortex from normal (norm), PD (PD), DLB brains (DLB), and pons of MSA brain (MSA). Equal amounts of sample were analyzed by SDS-polyacrylamide gel electrophoresis. These results show that the FA fraction from PD, DLB, and MSA brains harbors accumulations of oligomeric α-syn species, while variably increased amounts of Hsp90 and Hsc70, but not Hsp70, are seen in FA fractions of all α-synucleinopathy disease brains. Additionally, some pathological accumulations of Hsp40 as well as Hsp27 and αB-crystallin were noted in PD/DLB and MSA brains. Polyubiquitinated protein species in the FA fraction were more apparent in the disease brains than normal brain.

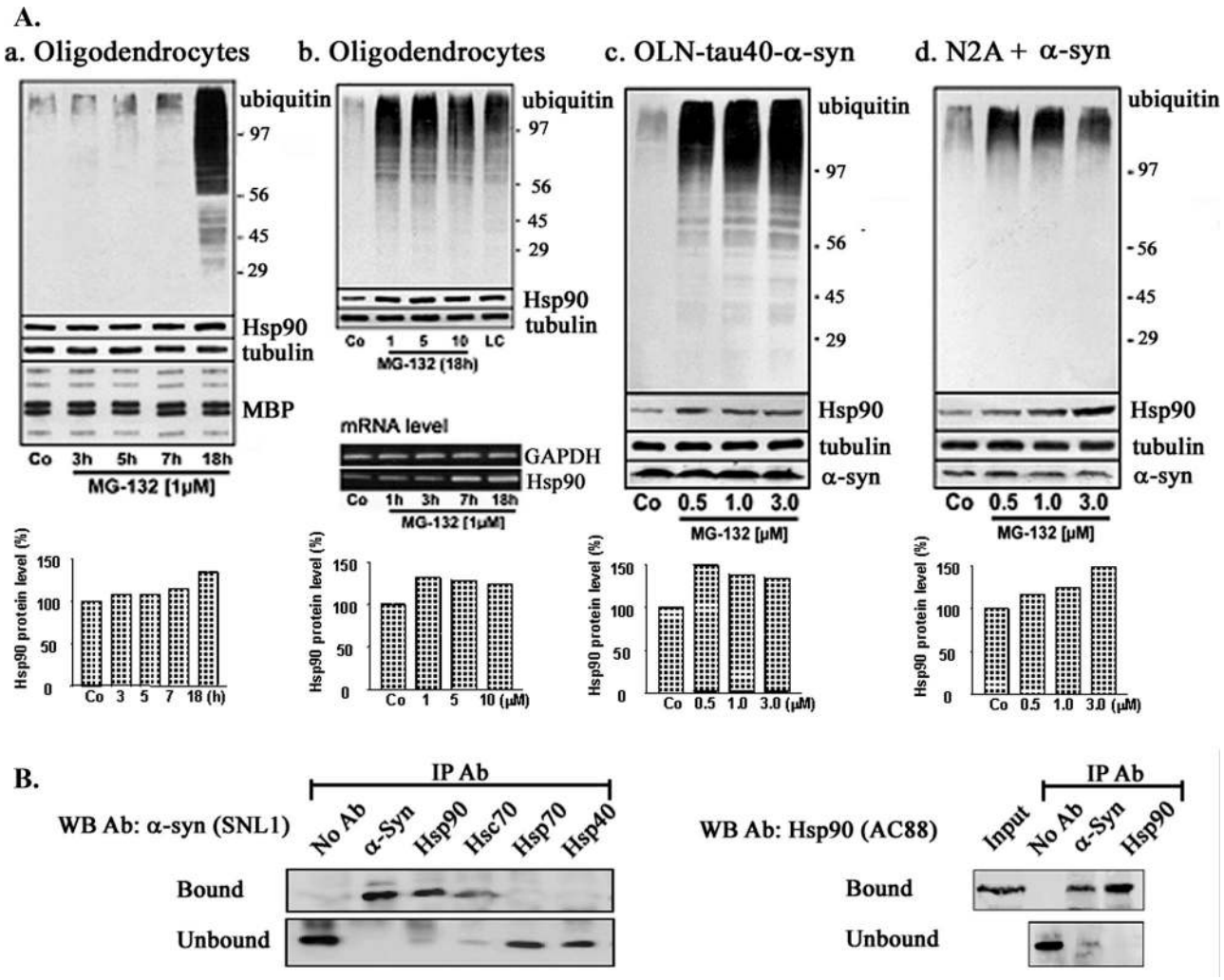

Figure 5. A: Effects of MG-132 and lactacystin (LC) on cultured cells. In oligodendrocyte primary culture (**a** and **b**), exposure to MG-132 (1 μmol/L) for 3, 5, 7, and 18 hours leads to the appearance of polyubiquitinated proteins after 18 hours, with slight up-regulation of Hsp90 levels (**a**). By 18 hours of incubation of different doses of MG-132 (1, 5, 10 μmol/L) or LC at one (10 μmol/L) dose there is a sharp induction of ubiquitinated proteins, accompanied by up-regulation of Hsp90 protein and mRNA level (**b**, **bottom**). Similar results are seen in OLN (**c**) cells transfected with tau40 plus α-syn and N2A cells (**d**) transfected with α-syn although the levels of tubulin and α-syn remain unchanged. Bar graphs indicate quantitation of variable Hsp90 protein levels due to MG132 treatment in respective cell types, confirming up-regulation of the protein level in all cases. **B:** Co-immunoprecipitation analysis using OLN-tau40-α-syn cells revealed noticeable protein-protein interactions between α-syn and Hsp90 as well as Hsc70 (bound), but not between α-syn and Hsp70 or Hsp40 (unbound). The protein complex of interest was isolated from the OLN cell homogenate using either no antibody (no Ab), α-syn Ab, Hsp90, Hsc70, Hsp70, or Hsp40 (IP Ab) and detected by SNL1 (α-syn) or AC88 (Hsp90) by Western blot (WB Ab). Input represents original material.

cific conditions used in these experiments. Notably, these data are in agreement with previous studies using oligodendrocytes or OLN cells without α-syn transfection, indicating that proteasome inhibition causes up-regulation of Hsps independent of the overexpression of α-syn.[27,60]

To test if Hsp90 induction by proteasomal inhibition is caused by the accumulation of nondegradable proteins or of increased transcription, total RNA was extracted from oligodendrocytes after various times of treatment with MG-132 (1 μmol/L). After reverse transcription, the resulting cDNA was subjected to polymerase chain reaction (reverse transcriptase-polymerase chain reaction). Reaction conditions were chosen so that amplification was in a linear range, and amplification of GAPDH was performed to control for equal loading of cDNA samples. Figure 5Ab (bottom) demonstrates that MG-132 caused an increase in mRNA encoding Hsp90.

Because mature primary oligodendrocytes contain very low levels of α-syn,[61] we performed another set of experiments using OLN-tau40-α-syn, representing a cell line with oligodendroglial characteristics,[27,62] and the N2A cell line after stable transfection to express human α-syn. Cells were treated with MG-132, and cell extracts were analyzed by Western blotting. Similar to primary rat brain oligodendrocytes, proteasomal inhibition by MG-132 led to a concentration-dependent increase in Hsp90 and ubiquitin in OLN-tau40-α-syn cells and N2A cells overexpressing α-syn; no effect on α-syn protein level was observed (Figure 5A, c and d). Hence, proteasome inhibition specifically leads to the recruitment of Hsp90 and ubiquitin (Figure 5A, c and d). Finally, co-immunoprecipitation analysis confirmed interactions between

α-syn and Hsp90 as well as Hsc70 in OLN cells (Figure 5B), but these immunoprecipitation methods did not demonstrate an association between α-syn and Hsp70 or Hsp40.

Hsp90 and α-Syn Pathology in α-Syn Tg Mice

Since previously established α-syn Tg mice develop LB-like α-syn inclusions that are ubiquitinated similar to human α-synucleinopathies,[19,63] this model was studied to determine whether these α-syn inclusions also sequestered Hsp90 like their human counterparts. IHC revealed that 3-month-old non-Tg mice (Figure 6, a–c), wild-type α-syn M7 Tg mice (not shown), and A53T mutant α-syn M83 Tg mice (asymptomatic) (Figure 6, d–f) had ubiquitous and modest expression of Hsp90 in neurons similar to normal human control brains (see Figure 1q). Among these lines of mice, only M83 Tg mice form α-syn lesions in an age-dependent manner. At 9 months of age, double-label FIHC revealed numerous α-syn IR inclusions in symptomatic M83 Tg mouse brain and co-localization of iHsp90 IR in a subgroup of these inclusions (Figure 6, g–i), while, in addition, a subset of these also were ubiquitinated (Figure 6, j–l). In contrast, the various control mouse lines showed unappreciable changes in α-syn, Hsp90, and ubiquitin IHC profiles between 3 and 9 month of age (data not shown). Further, Western blot analysis revealed the accumulation of Hsp90 and Hsc70 in the detergent insoluble FA soluble fraction selectively in symptomatic M83 Tg mouse brain, where aggregated α-syn and ubiquitinated protein species were detected (Figure 6, m–o). Other Hsps, including Hsp70, Hsp40, and αB-crystallin failed to show detectable levels in the same FA fractions.

Immunoelectron Microscopy of Hsp90 and α-Syn in Substantia Nigra LBs of PD

Postembedding double-immunoelectron microscopy studies were performed using anti-Hsp90 and anti-α-syn antibodies applied to midbrain sections, including substantia nigra (SN) dopaminergic neurons with LBs, of PD brains and visualized by 18-nm (Hsp90) and 10-nm (α-syn) protein gold. These studies confirmed co-localization of both proteins to the same bundles of filaments in LBs (Figure 7, a, c, and e), whereas neurons without α-syn inclusions revealed sporadic distribution of Hsp90-positive gold staining (Figure 7, b, d, and f). These data suggest that α-syn and Hsp90 are closely associated in α-syn protein inclusions.

Discussion

Filamentous intracytoplasmic inclusions are common pathological features of diverse neurodegenerative disorders, and these lesions are often associated with Hsps and ubiquitin,[64] as exemplified by α-synucleinopathies wherein α-syn fibrillizes to form ubiquitinated LBs, GCIs, and LNs.[63,65] Previous studies detected several different Hsps in LBs and other types of α-syn inclusions in diverse α-synucleinopathies, but Hsp70 has been the major focus of investigation.[18,31–33,66] However, we show that here among a large group of brain Hsps examined, Hsp90 most prominently and consistently co-localized in α-syn inclusions such as LBs, GCIs, LNs, many of which were ubiquitinated, as previously reported,[63] while Hsp90 also was most prominently associated with α-syn biochemically and with α-syn filaments ultrastructurally. Notably, Tg mouse models of α-synucleinopathies recapitulated this association between Hsp90 and α-syn pathologies, whereas cell culture studies suggested that impaired proteasome function may be causally linked to the accumulation of Hsp90, which then may interact with pathologically altered α-syn in the disease state. However, it is important to emphasize that although we show that here Hsp90 is the predominant Hsp linked to pathological α-syn in diverse α-synucleinopathies, other Hsps also are present in α-syn inclusions and pathologically altered thereby implicated them in these disorders as well. Significantly, by systematically assessing the differential involvement of multiple brain Hsps in α-syn pathologies, we provide a roadmap for focused next steps toward elucidating how Hsps, and especially Hsp90, contribute to the onset and/or progression of α-synucleinopathies.

Using a panel of different antibodies against a variety of Hsps, our data indicate that Hsp90 is the predominant Hsp sequestered in LBs, LNs, and GCIs throughout disease brains, but only rarely is Hsp90 found in tau inclusions. Furthermore, biochemical characterization of PD, DLB, and MSA brains and Tg mouse brains indicated that α-syn and Hsp90 shift into detergent insoluble fractions compared to control brains, whereas other Hsps (ie, Hsp70, Hsp40) did not. Thus, the predominant engagement of Hsp90 with α-syn in disease inclusions of α-synucleinopathies appears to be a selective process rather than the result of passive or nonspecific trapping of this Hsp. Although several studies implicate Hsp70 in α-syn pathogenesis,[18,31–33,66,67] other Hsps have not been as extensively examined in α-synucleinopathies and other neurodegenerative diseases. Thus, the predominant involvement of Hsp90 in mechanisms of α-synucleinopathies that we report here is substantiated by investigation of multiple Hsps in diverse α-synucleinopathies compared to other neurodegenerative disorders, as well as with a Tg mouse model of PD-like α-synucleinopathies. However, although Hsp90 is most abundant, this does not mean it is the most significant Hsp in these inclusions. The percentage of Hsp40- (~60%) and Hsp27-positive (~70%) LBs reported by McLean and colleagues[32] are similar to those we found here, whereas we found a much higher percentage of Hsp90 (95%) and lower percentage of Hsp70 (~5%) in LBs than McLean's report.[32] However, our data on Hsp70 are identical to those of Auluck and colleagues' report.[18] There are several factors that could explain the differences between our study and McLean and colleagues.[32] For example, we examined amygdala and McLean and colleagues[32] focused on substantia nigra, but other technical or methodological differences (eg, antibodies fixa-

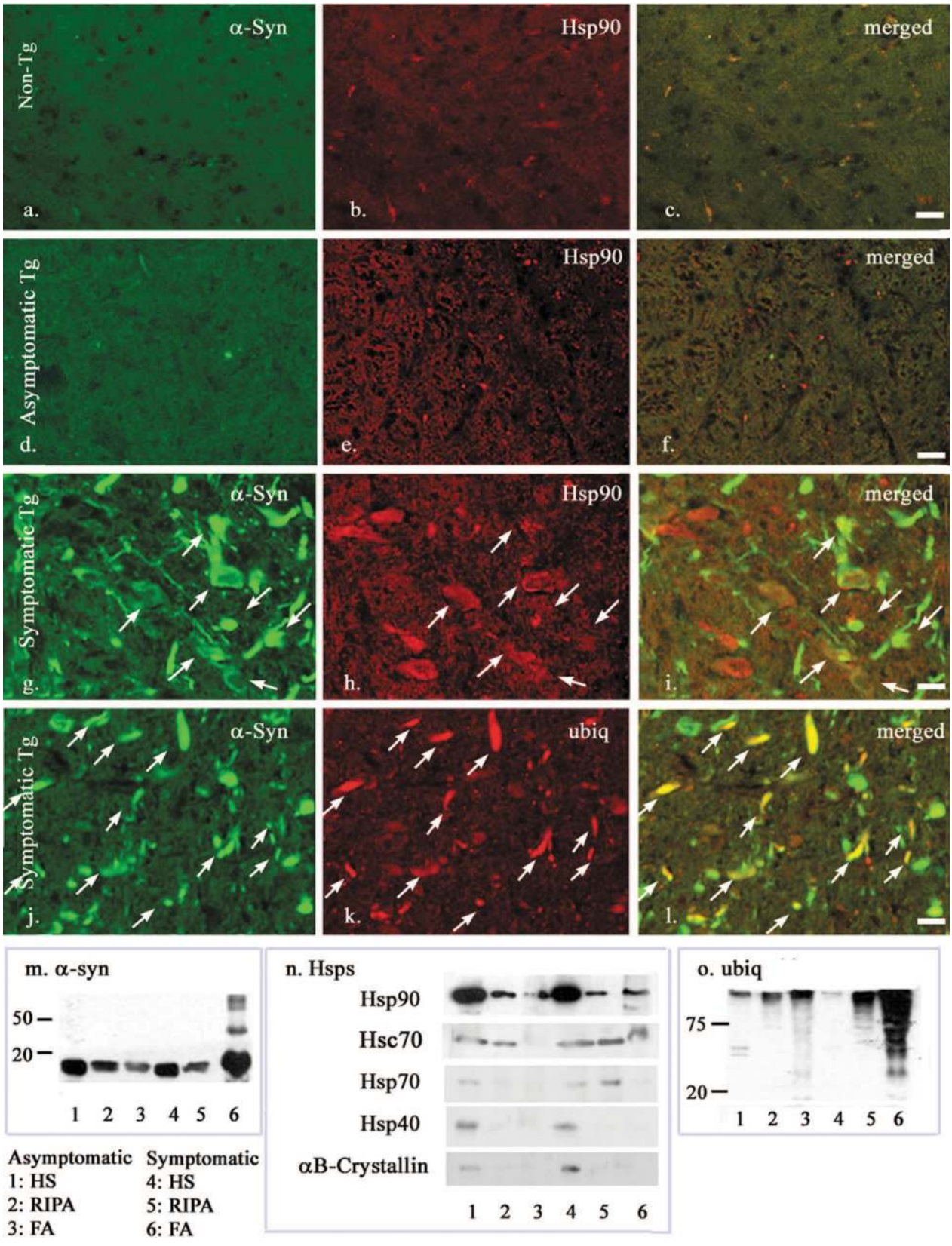

Figure 6. In the brainstem of a 3-month-old control non-Tg mouse (**a–c**), M7 Tg mouse (data not shown), and asymptomatic α-syn (M83) Tg mouse (**d–f**), there are no α-syn lesions and modest Hsp90 IR. However, in older symptomatic M83 Tg mouse (9 months old), numerous α-syn inclusions are formed, which are frequently associated with iHsp90 IR (**g–i**, **arrow**) and ubiquitin IR (**j–l**, **arrow**). Western blots of non-Tg (**lanes 1** to **3** in **m–o**) and M83Tg mouse (**lanes 4** to **6** in **m–o**) show accumulation of α-syn (**m**), Hsp90, and Hsc70, but not Hsp70, Hsp40, or αB-crystallin (**n**) and polyubiquitinated (**o**) proteins (likely including α-syn) exclusively in the FA fraction (**lane 6**) of 9-month-old symptomatic M83 Tg mouse brain, but not in the 3-month-old non-Tg mouse brain (**m–o**) or 9-month-old non-Tg mouse (data not shown). Scale bars, 10 μm (**a–l**).

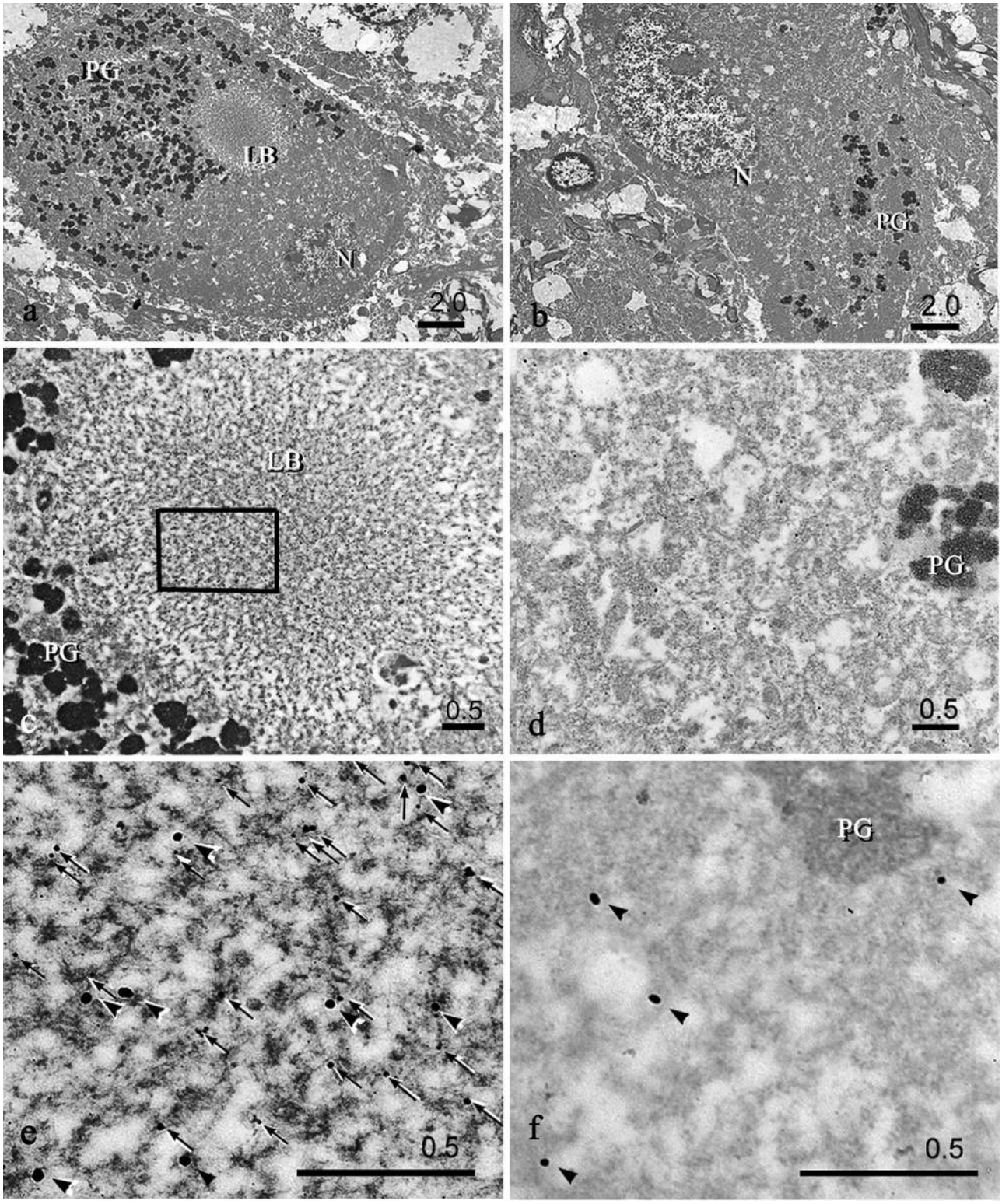

Figure 7. Double-immunoelectron microscopy reveals a close association of Hsp90 and α-syn in filamentous LBs in dopaminergic neurons of the SN in PD brain (**a**, **c**, **e**). α-Syn-positive (10 nm gold, **arrow**) filaments in the LB are also Hsp90-positive (18 nm gold, **arrowhead**), whereas unaffected dopaminergic neurons in the same SN section (**f**) reveal infrequent Hsp90 labeling (**arrowhead**). Image **e** is a high-power view of the **inset** in **c**, from the center of a LB. PG, neuronal pigment; N, nucleus.

tion, antigen retrieval) could also account for these differences.

Hsp90 is a highly conserved protein with chaperone activity.[30] It interacts with many proteins, including co-chaperones as well as substrates involved in signal transduction and cell cycle control. Hsp90 binds to these substrates, termed client proteins, and assists in final folding and stabilization. On stress, Hsp90 maintains

nonnative client proteins in a folding-competent structure and prevents irreversible denaturation, a process that requires ATP binding. When ATP is bound, Hsp90 may promote ubiquitination and degradation by directing client proteins to the proteasome.[68] Hence, Hsp90 is possibly involved in the balance between protein stabilization and degradation of its client proteins. Indeed, Hsps constitute a major defense against protein misfolding, and accumulation of misfolded proteins activates the ubiquitin/proteasome pathway,[69] whereas proteasome inhibitors up-regulate Hsps and ubiquitin.[25,27] Defects in the ubiquitin/proteasome pathway have been linked to PD and decreased proteasomal activity was reported in SN of sporadic PD.[70] Because Hsp90 may promote ubiquitination as well as target client proteins to the proteasome, it is plausible that Hsp90 is causally related to the ubiquitination of α-syn in fibrillary aggregates such as LBs and GCIs.

Understanding the molecular interactions among α-syn, Hsp90, and ubiquitin in α-syn pathogenesis is a question relevant to both biological and pathological processes, and our data suggest a variable co-localization among α-syn, Hsp90, and ubiquitin. The explanation for this is not entirely clear, but it is plausible that when α-syn initially becomes denatured/aggregated by stress caused by genetic or environmental factors, Hsp90 may engage α-syn and successfully rescue α-syn from further denaturing processes at this step. However, if stress persists chronically, α-syn may eventually attain a firmly aggregated stage, wherein Hsp90 fails to rescue α-syn from misfolding. Subsequently Hsp90 may redirect the protein to the proteasome system by facilitating its ubiquitination for degradation. Alternatively, Hsp90 may engage α-syn preferably when it becomes denatured and/or aggregated, to promote its degradation by facilitating ubiquitination. These speculations notwithstanding, future studies would clarify the nature of this pathological cascade. However, the presence of Hsps and ubiquitin in inclusions points to an unsuccessful attempt to remove aggregated proteins by the proteasomal machinery. As we show in cell culture systems here, proteasomal inhibition by MG-132 or lactacystin causes up-regulation of Hsp90, and leads to the accumulation of ubiquitinated proteins. Thus, a chronic impairment of the proteasomal system, which might occur insidiously during the onset or progression of neurodegenerative diseases, may not be counteracted by the induction of Hsps, and the increase and association of Hsp90 with α-syn aggregates might contribute to cell death.

In conclusion, our demonstration here of the predominant association of Hsp90 with ubiquitinated αyn inclusions in human α-synucleinopathies and animal models thereof, as well as a functional linkage between Hsp90 and proteasomal inhibition in cell culture models opens new avenues of investigation for elucidating the role of Hsps in mechanisms of neurodegenerative α-synucleinopathies.

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