Direct Transfer of α-Synuclein from Neuron to Astroglia Causes Inflammatory Responses in Synucleinopathies

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Abnormal neuronal aggregation of α-synuclein is implicated in the development of many neurological disorders, including Parkinson disease and dementia with Lewy bodies. Glial cells also show extensive α-synuclein pathology and may contribute to disease progression. However, the mechanism that produces the glial α-synuclein pathology and the interaction between neurons and glia in the disease-inflicted microenvironment remain unknown. Here, we show that α-synuclein proteins released from neuronal cells are taken up by astrocytes through endocytosis and form inclusion bodies. The glial accumulation of α-synuclein through the transmission of the neuronal protein was also demonstrated in a transgenic mouse model expressing human α-synuclein. Furthermore, astrocytes that were exposed to neuronal α-synuclein underwent changes in the gene expression profile reflecting an inflammatory response. Induction of pro-inflammatory cytokines and chemokines correlated with the extent of glial accumulation of α-synuclein. Together, these results suggest that astroglial α-synuclein pathology is produced by direct transmission of neuronal α-synuclein aggregates, causing inflammatory responses. This transmission step is thus an important mediator of pathogenic glial responses and could qualify as a new therapeutic target.

Parkinson disease (PD) is a complex neurodegenerative disease related to aging process. Clinically, it is characterized by a variety of motor and non-motor symptoms, and pathologically by selective neurodegeneration and the occurrence of Lewy bodies in central and peripheral nervous tissues (1). Evidence from genetic, pathologic, and biochemical studies suggests that abnormal deposition of the neuronal protein α-synuclein produces the pathogenesis of PD (2). Aggregated forms of α-synuclein are the major component of Lewy bodies (3), which are also found in other neurological diseases, such as dementia with Lewy bodies (DLB) and Alzheimer disease (AD). Furthermore, recent studies showed extensive deposition of filamentous α-synuclein aggregates in astroglia and oligodendrocytes in PD, DLB, and multiple system atrophy (4–8), even though these cells express very low levels of the protein (9–11). The mechanism of inclusion body formation in neurons and glia and the role in disease onset and progression remain largely unknown.

α-Synuclein is a cytosolic protein with no known signal sequence. Previously, we showed that small amounts of the protein, both overexpressed and endogenous, are present in the lumen of vesicles and secreted from neuronal cells by exocytosis (12). Both monomeric and the aggregated forms were found in vesicles and exocytosed. Monomeric and oligomeric forms of α-synuclein have been detected in human cerebrospinal fluid and blood plasma (13), and the levels of the oligomeric forms were elevated in PD patients compared with control cases (14). Together, these results suggest the pathogenic actions of α-synuclein extend to the extracellular space and neighboring cells. Consistent with this hypothesis, it was shown that extracellular exposure of cultured microglia to aggregated or nitrated α-synuclein produced some signs of activated microglia (15, 16). More recently, it was shown that α-synuclein aggregates released from neuronal cells can be transferred to neighboring neurons forming Lewy-like inclusions (17), providing mechanistic basis for the development of Lewy pathology in mesencephalic transplants in PD patients (18, 19). Despite these exciting new findings, the pathophysiological roles of the secreted α-synuclein remain largely unclear.

Here, we report that α-synuclein proteins released from neuronal cells are readily endocytosed by astrocytes. When the internalized α-synuclein accumulates in astrocytes, the cells produce glial inclusions and inflammatory responses. Therefore, when neurons are under protein conformational stresses forming α-synuclein aggregates, secreted forms of α-synuclein...
potentially act as a messenger between neurons and astroglia, inducing neuroinflammatory responses.

**EXPERIMENTAL PROCEDURES**

**Materials—**All-trans-retinoic acid, proteinase K, Triton X-100, thioflavin S, and protease inhibitor mixture were purchased from Sigma. Bafilomycin A1 was purchased from Calbiochem (San Diego, CA). The Dynamin K44A/pCB1 vector was a kind gift from Dr. Marc Caron. Recombinant human wild-type α-synuclein was purchased from ATGen (Sunnam, Korea). The following primary antibodies were used: α-synuclein monoclonal antibody (Cell Signaling Technology, Beverly, MA), α-synuclein monoclonal antibody (BD Biosciences, San Diego, CA), Myc polyclonal antibody (Abcam, Cambridge, MA), anti-dynamin antibody (BD Biosciences), anti-GFP N terminus antibody (Cell Signaling Technology), anti-GFAP antibody (Abcam), anti-S100 antibody (Abcam), anti-GLUT1 antibody (Abcam), anti-Hsp70/Hsc70 antibody (Stressgen, Victoria, British Columbia, Canada), anti-20S antibody (Calbiochem), anti-ubiquitin antibody (Chemicon, Temecula, CA), anti-IL-1α antibody (Abcam), anti-IL-1β antibody (Abcam), anti-IL-6 antibody (Abcam), and anti-β-actin antibody. A monoclonal antibody for LAMP 2 (H4B4) developed by Drs. August and Hildreth and a recombinant adenoviral vector containing human α-synuclein cDNA (adeno/α-syn) were previously described (21) and maintained in Dulbecco’s modified Eagle’s medium/F-12 medium (Invitrogen) with 10% fetal bovine serum (HyClone Laboratories) and penicillin/streptomycin (Invitrogen). Briefly, dissected cortices without meninges were broken into smaller pieces by pipetting in Dulbecco’s modified Eagle’s medium/F-12 medium. After treatment with DNaseI digestion, medium was added to inactivated trypsin, and cells were centrifuged at 500 g. After overnight incubation at 37 °C, the conditioned medium was centrifuged at 1,000 × g for 10 min, and the supernatant was removed further at 10,000 × g to remove debris and dead cells. The recovered supernatant was concentrated 50- to 100-fold using Amicon 10K MWCO filters (Millipore, Billerica, MA). The concentrations of non-tagged and Myc-tagged α-synuclein in the conditioned medium measured by enzyme-linked immunosorbent assay were 4.23 ± 1.2 µg/ml and 83.1 ± 27.3 ng/ml, respectively. The concentrations of α-synuclein in the media of rat primary neuron cultures were measured by quantitative Western blotting with recombinant human α-synuclein standards and were approximately ~3 ng/ml.

**Depletion of α-Synuclein from Conditioned Medium—**The conditioned medium from polyhistidine-tagged α-synuclein-overexpressing SH-SY5Y cells was prepared as described above. The conditioned medium was incubated with Talon beads (BD Biosciences) to deplete α-synuclein, and the beads were removed by centrifugation.

**Western Blotting—**Western blotting was performed using a monoclonal anti-α-synuclein antibody (Syn-1, BD Biosciences) as previously described (22). Images were obtained and quantified using a luminescent image analyzer LAS-3000 (Fujifilm, Japan) and Multi Gauge (v3.0) software.

**α-Synuclein Transgenic Mice and Treatments—**For this study, a total of 12 heterozygous transgenic (tg) mice (Line D) expressing human α-synuclein under the regulatory control of the platelet-derived growth factor-β (PDGFB) promoter (23) (6 months old) and 12 non-transgenic age-matched littermates were used. These animals were selected because they display abnormal accumulation of detergent-insoluble α-synuclein and develop α-synuclein-immunoreactive inclusion-like structures in the brain. Although some nuclear staining has been observed in this model, distinct cytoplasmic inclusion-like structures have been consistently identified by confocal and electron microscopy (23, 24). Furthermore, these animals also display neurodegenerative and motor deficits that mimic certain aspects of PD and DLB. Comparisons for the patterns of α-synuclein distribution were performed in the brains of our model mimicking AD-like pathology by expressing the human α-synuclein cDNA.
mutated amyloid precursor protein (APP tg, line 41) (n = 6, 6 months old) under the thy1 promoter (25). In addition, studies of the re-distribution of α-synuclein were performed in the PDGFα-syn-GFP tg mouse model (line 78) (n = 6, 6 months old) (26), because in these mice the fused GFP protein expected to reduce the neuronal release of α-synuclein compared the PDGFα-syn tg lines (line D). Animal protocols were approved by the University of California San Diego Institutional Animal Use and Care Committee.

Cases and Neuropathological Evaluation—For this study we included a total of 18 cases (for detail see supplemental Table S4); of these, 4 were non-demented controls, 8 were DLB cases, and the other 6 were AD cases. Autopsy material was obtained from patients studied neurologically and psychologically at the Alzheimer Disease Research Center/University of California, San Diego.

For each case, paraffin sections from 10% buffered formalin-fixed neocortical, limbic system, and subcortical material stained with hematoxylin and eosin and thioflavin-S were used for routine neuropathological analysis (27) that included Braak stage (28). The diagnosis of DLB was based on the clinical presentation of dementia and the pathological findings of Lewy bodies in the locus coeruleus, substantia nigra, or nucleus basalis of Meynert, as well as in cortical and subcortical regions. Lewy bodies were detected using hematoxylin and eosin anti-ubiquitin and anti-α-synuclein antibodies as recommended by the Consortium on DLB criteria for a pathologic diagnosis of DLB (29). In addition to the presence of Lewy bodies, the great majority of these cases display sufficient plaques and tangles to be classified as Braak stages III–IV. Specifically, DLB cases had abundant plaques in the neocortex and limbic system but fewer tangles compared with AD cases.

**Immunohistochemistry and Laser Scanning Confocal Microscopy**—The procedure for immunofluorescence cell staining has been described elsewhere (22). Nuclei were stained with TOPRO-3 dye (Invitrogen), and the coverslips were mounted on slides in Antifade reagent (Invitrogen). Cells were observed with an Olympus FV1000 confocal laser scanning microscope. For three-dimensional analysis of images, serial images through z axis were obtained and analyzed using FV10-ASW software (Olympus).

For immunohistochemistry of tissue sections, free-floating 40-μm-thick vibratome sections were washed with Tris-buffered saline (pH 7.4), pre-treated in 3% H₂O₂, and blocked with 10% serum (Vector), 3% bovine serum albumin (Sigma), and 0.2% gelatin in Tris-buffered saline-Triton X-100. Sections were incubated at 4 °C overnight with the rabbit polyclonal antibody against human α-synuclein (Chemicon), and then incubated with secondary antibody (1:75, Vector Laboratories, Burlingame, CA), followed by avidin biotin horseradish peroxidase (ABC Elite, Vector) and reacted with diaminobenzidine (0.2 mg/ml) in 50 mm Tris (pH 7.4) with 0.001% H₂O₂. Control experiments consisted of incubation with pre-immune rabbit serum. Double-immunofluorescence analyses of tissue sections were performed utilizing the Tyramide Signal Amplification™ Direct (Red) system (PerkinElmer Life Sciences). Specificity of this system was tested by deleting each primary antibody. For this purpose, sections were double labeled with the monoclonal antibodies against α-synuclein (1:20,000, Cell Signaling) detected with Tyramide Red and either S100 detected with fluorescein isothiocyanate-conjugated secondary antibodies (1:75, Vector). All sections were processed simultaneously under the same conditions, and experiments were performed twice for reproducibility. Sections were imaged with a Zeiss 63× objective on an Axiosvert 35 microscope (Zeiss, Germany) with an attached MRC1024 laser scanning confocal microscope system (Bio-Rad).

**In Situ Hybridization—In situ** hybridization was carried out using human α-synuclein sense and antisense Riboprobes labeled with ³⁵S, essentially as described previously (30). Briefly, paraffin sections were treated with Proteinase K, incubated in the prehybridization solution, and hybridized with Riboprobes at 60 °C. Sections were exposed to film and developed 3 days to 2 weeks later. These sections were then dipped in Kodak NTB-2 emulsion and developed 10 days later.

**RNA Preparation and RT-PCR**—Total RNA was prepared from primary astrocytes using the RNeasy mini kit (Qiagen). The RNA was used for microarray and RT-PCR analysis. For RT-PCR, 2 µg of total RNA was used to synthesize cDNA using iScript cDNA synthesis kit (Bio-Rad) following the manufacturer’s protocol. Two microliters of synthesized cDNA was used for PCR using ImmomixRed (Bioline) with primers and reaction temperatures as indicated in supplemental Table 3.

**Cytokine Assay**—Rat 8-plex cytokine kit was obtained from Panomics (Fremont, CA). Primary astrocytes grown in a 12-well plate were treated with LacZ or α-synuclein conditioned medium for 24 h. The medium from astrocytes were collected and centrifuged at 10,000 × g for 20 min, and the supernatant was obtained. Quantification of TNFα and CXCL1 was performed according to the manufacturer’s instructions. The result was read using a Bio-Plex 200 system (Bio-Rad).

**Microarray Analysis**—The integrity of the total RNA extracted from primary astrocytes was assessed using a Bioanalyzer 2100 (Agilent, Santa Clara, CA), and the RNA integrity numbers were 10 for all samples. 500 ng of high quality total RNA was amplified with a TotalPrep amplification kit (Ambion, Austin, TX), and 750 ng of amplified cRNA was hybridized to the RatRef-12 expression BeadChip for 16 h. The hybridized array was scanned with a BeadArray Reader (Illumina). The microarray data are deposited in a GEO data base under accession number GSE11574.

**Statistical Analysis of the Microarray Data**—Using the Illumina RatRef-12 Expression BeadChip, we obtained expression levels for nearly 22,000 genes in astrocytes exposed for 6 and 24 h to conditioned media from cultures of α-synuclein and lacZ-expressing cells. There were three array replicates done for each condition (astrocytes treated for 6 and 24 h with lacZ and α-synuclein containing conditioned media). The probe intensities were normalized at log2 scale using quantile normalization in BeadArray 1.6, an R/Bioconductor package. Probe annotation was done using lumi 1.4, an R/Bioconductor package providing an integrated solution for Illumina microarray data analysis. Before identifying differentially expressed genes, we first applied a Gaussian mixture modeling technique to the normalized data to determine present/absent calls as follows. In the mixture modeling, 1) two Gaussian probability density
functions, one for absent and the other for present probes, were fitted to the distribution of the observed probe intensity and 2) among the 22,523 probes, 11,039 probes, whose maximum intensities in all 12 samples were higher than the threshold intensity, where two fitted Gaussian probability density functions meet, were determined to be present.

Then, we identified differentially expressed genes (DEGs) between astrocytes treated with α-synuclein and lacZ containing conditioned media independently for two time points (6 and 24 h) by an integrative statistical test combining the p values from three statistical hypothesis testing methods: Student two-tailed t test, Wilcoxon rank-sum test, and median difference test. In each test, an empirical null probability density function was estimated for the corresponding statistic (T, rank sum, and median difference) computed from all possible permutations of the samples. A p value for the observed statistic of each probe was then computed using the empirical probability density function. For each probe, p values resulting from three statistical tests were combined as an overall p value using meta analysis (Stouffer’s method) as described in Hwang et al. (31). DEGs were selected as the genes with overall p values of <0.01. Also, false positives from potential bias toward p values from particular hypothesis testing in meta-analysis were reduced by removing genes that showed -fold differences < 1.5 between samples treated with α-synuclein and lacZ containing conditioned media. We performed a functional enrichment analysis to associate the DEGs with GO Biological Processes (GOBPs), using BiNGO, a Cytoscape Plugin, and KEGG pathways, using the Database for Annotation, Visualization and Integrated Discovery (see Ref. 32). Several GOBPs and KEGG pathways statistically enriched (p < 0.05) by the DEGs were identified (see supplemental Tables 1 and 2). Finally, network analysis was done using Cytoscape (33) with information of the DEGs associated with GOBPs and KEGG pathways related to inflammatory responses and known protein-protein interactions from BIND and HPRD (both available on-line). Human and mouse protein-protein interactions were translated into those in rat via ortholog ID mapping using ortholog information in the Mouse Genome Informatics (MGI) data base (available on-line).

**Statistical Analyses of Quantified Data**—Values in the figures are expressed as means ± S.E. Differences were considered significant if p values were <0.05. For determination of statistical significance, values were compared by one-way analysis of variance with Tukey’s post test or Student’s t test using GraphPad InStat version 1.01 GraphPad software. The correlation coefficients (Pearson r) and the p values were determined by linear regression analysis.

**FIGURE 1.** Uptake of neuronal cell-derived α-synuclein from conditioned medium by astrocytes. A, Western analysis of α-synuclein uptake in primary astrocytes. α-Synuclein-containing conditioned medium was obtained from SH-SYSY culture and added to the primary astrocytes for the indicated times. Tx-s, Triton X-100-soluble; Tx-in, Triton X-100-insoluble. B, immunofluorescence staining of primary astrocytes (GLUT1-positive) treated with the conditioned media used in C. Top panels: LacZ medium. Middle panels: Myc-His-tagged α-synuclein (α-synMH) medium. Bottom panels: depleted medium. A perinuclear inclusion body produced by the internalized α-synuclein is indicated by an arrow in the middle panel. Scale bars: 20 μm. Experiments were repeated three times. C, depletion of α-synMH from the conditioned medium of differentiated SH-SYSTY cells expressing α-synMH. D, uptake of neuronal cell-derived α-synuclein by astrocytes. Conditioned media from LacZ- or α-synMH-expressing cells, or the latter with α-synMH depleted were incubated with rat astrocytes for the indicated times. Note that both monomeric and aggregated forms (represented by the smear between the 37- and 180-kDa markers) of α-synuclein were taken up from the α-synMH medium.
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FIGURE 2. Cell-to-cell transmission of \( \alpha \)-synuclein to primary astrocytes in co-culture. A and B, primary astrocytes were co-cultured with differentiated SH-SYSY cells overexpressing \( \alpha \)-synuclein. Serial images through \( z \) axis were obtained and analyzed to show that transmitted \( \alpha \)-synuclein (green) reside inside of astrocytes (red: GFAP). Areas in the white boxes are enlarged (left panels, \( xy \) images; right panels, \( z \)-stacked images seen through the \( y \) axis). C, transfer of neuronal endogenous \( \alpha \)-synuclein to astrocytes. Rat primary cortical neurons were co-cultured with astrocytes. Note that the \( \alpha \)-synuclein polyclonal antibody used in this experiment also recognizes rat sequences. Top panels: primary astrocytes cultured with neurons. Bottom panels: primary astrocytes only. White perforated lines indicate outlines of astrocyte periphery. D, primary astrocytes were co-cultured with differentiated SH-SYSY cells overexpressing \( \alpha \)-synuclein and fixed at the indicated times. The control indicates a co-culture with SH-SYSY cells without \( \alpha \)-synuclein overexpression. Arrows: perinuclear inclusions. E, quantitative analyses of \( \alpha \)-synuclein transfer (left graph) and glial inclusion formation (right graph). Differentiated SH-SYSY cells overexpressing human \( \alpha \)-synuclein were co-cultured with rat primary astrocytes. The transfer was measured by fluorescence intensities of \( \alpha \)-synuclein staining in astrocytes, and the inclusion formation was measured by the percentage of astrocytes with inclusions among the cells with transferred \( \alpha \)-synuclein. Experiments were repeated four times, and more than hundred cells were counted per slide. All scale bars, 20 \( \mu \)m (*, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \)).

RESULTS

Transmission of \( \alpha \)-Synuclein to Astrocytes and Inclusion Body Formation—Recent studies show that cytoplasmic \( \alpha \)-synuclein deposition occurs in both neurons and astrocytes in PD (4, 6). Considering the fact that astrocytes express no or low levels of \( \alpha \)-synuclein on their own and are able to take up recombinant \( \alpha \)-synuclein aggregates from the culture media (34), we hypothesized that neuronal \( \alpha \)-synuclein aggregates are transmitted to adjacent astrocytes and form pathological inclusion bodies. To test this hypothesis, the conditioned medium containing secreted \( \alpha \)-synuclein from differentiated SH-SY5Y cells was added to primary astrocytes. The secreted \( \alpha \)-synuclein was readily internalized by the astrocytes, as judged by Western blotting (Fig. 1A) and immunofluorescence microscopy (Fig. 1B). The presence of \( \alpha \)-synuclein inside astrocytes was verified by proteinase K resistance (supplemental Fig. S1). Internalized proteins were found in both the Triton-soluble and -insoluble fractions, and a portion of these appeared as SDS-stable high molecular weight complexes (Fig. 1A and supplemental Fig. S2), suggesting internalized \( \alpha \)-synuclein maintains its aberrant conformation after internalization. Some astrocytes accumulated the internalized \( \alpha \)-synuclein in large juxtanuclear inclusion bodies (Fig. 1B). Approximately 30% of cells took up \( \alpha \)-synuclein, and \( \sim 4.6\% \) of these cells showed large inclusion bodies. As a control, when \( \alpha \)-synuclein was depleted from the conditioned medium (Fig. 1C), this protein was not detected in astrocytes (Fig. 1, B and D). These results exclude the possibility of transmission of \( \alpha \)-synuclein vector to the astrocytes and demonstrate that neuronal cell-derived \( \alpha \)-synuclein can be taken up by astrocytes and accumulate in inclusion bodies.

Next, transmission of \( \alpha \)-synuclein from neuronal cells to astrocytes was directly demonstrated in a coculture system consisting of primary rat astrocytes and differentiated SH-SYSY cells expressing human \( \alpha \)-synuclein (Fig. 2 and supplemental Fig. S3). Antibodies specific to the human sequence of \( \alpha \)-synuclein enabled detection of neuronal \( \alpha \)-synuclein only, and the rat astrocytes were identified by the markers GFAP, GLUT1, and S100. In this co-culture, human \( \alpha \)-synuclein was observed in the rat astrocytes, consistent with transmission of \( \alpha \)-synuclein from the neuroblastoma (supplemental Fig. S3). Z-stack imaging of these cells confirmed the presence of internalized \( \alpha \)-synuclein in the astroglial cytoplasm (Fig. 2, A and B). Co-culture of rat astrocytes with SH-SY5Y cells expressing LacZ gene did not show \( \alpha \)-synuclein accumulation in astrocytes (supplemental Fig. S3D). Transmission of endogenous neuronal \( \alpha \)-synuclein from the primary cortical neurons to astrocytes was also demonstrated (Fig. 2C).

The transfer was time-dependent and widespread; by day 5 of co-culture, \( \sim 80\% \) of astrocytes received neuronal cell-derived \( \alpha \)-synuclein (Fig. 2, D and E, and supplemental Fig. S3E). Changes in the distribution of internalized \( \alpha \)-synuclein were also observed over time. Initially, the internalized \( \alpha \)-synuclein was distributed in vesicular patterns, which evolved to larger punctates and finally showed large perinuclear inclusions. Among the \( \alpha \)-synuclein-positive astrocytes, up to 10% of cells developed round inclusion bodies (Fig. 2E). Expression of \( \alpha \)-synuclein in SH-SY5Y neuroblastoma cells was maintained during this 5-day period (supplemental Fig. S4).

The \( \alpha \)-synuclein-positive perinuclear inclusions shared some characteristics with Lewy bodies and PD-associated astroglial inclusions (Fig. 3). More than 90% of the astrocyte perinuclear inclusions were stained with thioflavin S, an indicator of fibrillar aggregates (Fig. 3, A and E). A large proportion of these inclusions contained some of the proteins found in

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Lewy bodies, such as ubiquitin and the 20 S proteasome α-subunit, and to a lesser degree, Hsp/Hsc70 (Fig. 3, A–C). These results suggest that neuron-to-astrocyte transmission of aberrant α-synuclein may be a mechanism of astroglial α-synuclein deposition in PD and other Lewy body diseases.

To determine if the transfer involved endocytosis, astrocytes were transfected with the dominant negative dynamin-1 K44A mutant, which inhibits endocytic vesicle formation (35), and co-cultured with SH-SY5Y cells expressing α-synuclein (Fig. 4, A–C). The α-synuclein fluorescence intensity in astrocytes was reduced ~4-fold in dynamin-1 K44A-expressing cells, compared with non-transfected cells, consistent with endocytosis-mediated α-synuclein transmission. As a control, GFP transfection had no effect on α-synuclein transmission (supplemental Fig. S5). Further analysis of internalized α-synuclein showed that most of the α-synuclein co-localized with the lysosome marker LAMP-2 (Fig. 4D). This indicates that internalized α-synuclein moves through the endosomal pathway and is normally destined to be degraded in the lysosome.

Neuron-to-Glia α-Synuclein Transmission and Astroglial Inclusion Body Formation in Vivo—To verify the neuron-to-astrocyte transmission in a physiological context, we examined the brain sections of tg mice overexpressing human α-synuclein under neuronal promoter (PDGF). In non-tg controls and PDGF-APP tg mice (see “Experimental Procedures”), α-synuclein immunolabeling was localized to the presynaptic terminals in the neuropil (Fig. 5A). Although abundant glial cells are present around the plaques in the APP tg model, α-synuclein accumulated in the dystrophic neurites but not in astroglial cells. In the PDGFα-syn tg mice, abundant α-synuclein accumulation was observed not only in neurons but also in glial cells (Fig. 5A). This glial accumulation of α-synuclein was more abundant in the deeper layers of the cortex and along the myelinated axons. The accumulation of α-synuclein in astroglial cells was considerably suppressed in PDGFα-syn-green fluorescent protein (GFP) tg mice (Fig. 5, A and B). In this line, most of the α-synuclein labeling was restricted to axons and neuronal cell bodies. This is consistent with our in vitro studies showing that tagging α-synuclein with a variant of GFP reduces the secretion of α-synuclein.3 Previously, we have shown that, unlike

3 A. Jang, H.-J. Lee, J.-E. Suk, J.-W. Jung, K.-P. Kim, and S.-J. Lee, submitted for publication.
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A

Non tg

APP tg

α-syn tg

α-syn GFP tg

β-syn tg

B

α-syn

S100

merged

DLB

α-syn

S100

merged

merged

C

neurons in neocortex

neurons in hippocampus

glial cells in colliculus

D

FIGURE 5. In vivo evidence for neuron-to-glia transmission of α-synuclein in tg models. A, immunocytochemical distribution of α-synuclein in the neocortical deeper layers in non-tg control, PDGF-APP tg, PDGF-α-syn tg, PDGF-α-syn-GFP, and β-syn tg. In the APP tg mice α-synuclein immunoreactivity is localized in dystrophic neurites around the plaque (arrow), whereas in the PDGF-α-syn tg, α-synuclein accumulates both in neuronal (arrows) and glial cells (*). No glial accumulation of α-synuclein was detected in PDGF-α-syn-GFP and β-syn tg. Experiments were performed in triplicate, and the representative images are shown. B, double labeling and confocal microscopy analysis, showing that, in DLB cases as well as in PDGF-α-syn tg mice, glial cells expressing S100 displayed human α-synuclein accumulation (arrows). In contrast, in the PDGF-α-syn-GFP tg mice no GFP signal (associated with α-synuclein) was detected in S100-positive cells. At least three independent experiments were performed, and the representative images are shown. C, in situ hybridization for the detection of human α-synuclein mRNA. Human α-synuclein is expressed mostly in neurons (top and middle panels). D, quantification of data represented in B (top panel) and C (bottom panel). For counting of glial cells with accumulated α-synuclein (top panel), PDGF-α-syn tg mice (n = 6), PDGF-α-syn-GFP mice (n = 4), DLB cases (n = 8), and control cases (n = 6) were analyzed. The graph on the bottom panel presents % cells containing human α-synuclein mRNA. Six mice of each group were analyzed. For each mouse 2 sections were evaluated, and from each section 5 fields, on average, 75–100 cells were counted. White bars, NeuN-positive neurons; black bars, S100-positive astrocytes. Scale bars, 20 μm (**, p < 0.01; ***, p < 0.001).

α-synuclein, little β-synuclein was secreted from cells (12). Consistently, in β-synuclein tg mice, both the endogenous α-synuclein and β-synuclein were exclusively localized to the neurons and no glial immunoreactivity was detected (Fig. 5A).

Similar to the findings in the mouse models, α-synuclein immunoreactivity in the temporal cortex from human control and AD cases was present in the presynaptic terminals in the neuropil and in dystrophic neurites around the plaques, respectively (supplemental Fig. S6). In contrast, in DLB cases α-synuclein immunoreactivity was present in the Lewy bodies and astroglial cells in the deeper layers of the neocortex and the white-gray matter junction. Double labeling and confocal microscopy studies confirmed that both in DLB cases as well as in PDGFα-syn tg mice, glial cells expressing S100 and GFP displayed α-synuclein accumulation (Fig. 5, B and D). In contrast, no α-synuclein was detected in astrocytes in the PDGFα-syn-GFP tg mice. To rule out the possibility of transgene expression in astrocytes in PDGFα-syn tg mice, the cell type distribution of human α-synuclein mRNAs were examined by in situ hybridization. Consistent with the use of neuron-specific promoter, human α-synuclein mRNAs were found mostly in neurons, not in glial cells (Fig. 5, C and D). Together, these results verify that astroglial inclusion bodies are formed by the accumulation of α-synuclein proteins transmitted from neurons.

Gene Expression Changes in Astrocytes—To determine how astrocytes respond to extracellular α-synuclein, gene expression profiles were established and analyzed for nearly 22,000 rat genes using the Illumina RatRef-12 Expression BeadChip. Total RNA was isolated from astrocytes exposed for 6 or 24 h to conditioned medium from cultures of α-synuclein or lacZ-expressing cells. About 1,116 DEGs were identified (supplemental Fig. S7). The expression of about half of these genes was up-regulated (red), whereas the other half showed down-regulation (Fig. 6A). Many inflammatory cytokines, such as IL-1α, -1β, and -6, and CC, CXC, CX3C-type chemokines, showed increased expression. These cytokines typically are involved in the inflammatory responses in the central nervous system. Other DEGs are involved in antigen processing and presentation, toll-like receptor signaling, apoptosis, mitogen-activated protein kinase signaling, the cell cycle, complement cascade, and leukocyte transendothelial migration, and many of these are important for brain immune responses (see supplemental Tables 1 and 2 and supplemental Fig. S8).

To confirm the inflammatory response, we constructed a hypothetical protein network (Fig. 6B), using the inflammation-related DEGs from the current study and information about protein-protein interactions from BIND and HPRD. The network node proteins were clustered into several network modules that represent the enriched GOBP terms and enriched KEGG pathways. The astroglial protein network was perturbed by exposure to neuronal cell-derived α-synuclein and showed
up-regulation of several cellular processes associated with inflammation and innate immunity. The modules involved in this network include Toll-like receptor signaling, cytokines and chemokines, antigen processing and presentation, cell migration, and the JAK-STAT pathway. Based on this network, in astrocytes exposed to neuron-derived α-synuclein aggregates, Toll-like receptor signaling appears to be activated, as evidenced by the increased expression of TLR2, CD14, NFκB1/2, and RELA. Activated NF-κB complexes potentially induce the transcription of several cytokines and chemokines (IL1B, IL6, CCL3/4/5, and CXCL4/10/11/12) (36), which in turn activate representative components of the inflammation and innate immunity-related modules. These include: (i) antigen processing and presentation (PSME1/2, TAP1/2, TAPBP, CD74, CTSB, and MHC1/II), (ii) cell migration, (ICAM1, VCAM1, CXCL12, NCF1C, CYBA, and MMP9), and (iii) the JAK-STAT pathway activated by the up-regulated interleukins (37–40).
Neuron-to-Astroglia Transmission of α-Synuclein

The gene expression changes were verified by RT-PCR analysis of key genes of the functional modules. The results confirm up-regulation of cytokines such as IL-1, IL-6, and TNFα, chemokines CCL2, CCL20, CXCL1, and C3X3L1, cell adhesion molecules ICAM1 and VCAM1, metalloproteinases MMP-3 and -9, proteins in Toll-like receptor signaling (TLR-2 and NFκB), Fc receptor signaling (FCGR3), and antigen processing (TAPBP and CD74) (Fig. 6C). Down-regulation of TGF-β3 and CXCR4 was also confirmed by RT-PCR. Increased expression of IL-6 and ICAM had previously been reported in primary astrocytes and astroglomas exposed to α-synuclein (41), consistent with our current results. Therefore, the network suggests that extracellular α-synuclein secreted from neuronal cells mediates neuron-astrocyte interaction, inducing neuroinflammatory responses in astrocytes.

The relationship between the cytokine production and glial α-synuclein accumulation was investigated by using quantitative fluorescence microscopy and the linear regression analysis. Production of cytokines, such as IL-1α, IL-1β, and -6, strongly correlated with uptake of the neuron-derived α-synuclein (Fig. 6D), with the correlation coefficients of 0.39 (p = 0.0036), 0.78 (p = 0.00004), and 0.75 (p = 0.0014), respectively. This suggests that the cytoplasmic accumulation of α-synuclein within the astrocytes is important for the inflammatory response, and that exposure to this protein is insufficient to elicit a response.

To further determine the relationship between α-synuclein accumulation and inflammatory responses in astrocytes, we used bafilomycin A1, a lysosomal inhibitor, to stabilize the internalized α-synuclein. Consistent with our previous finding that α-synuclein aggregates, after internalization, travel through the endocytic pathway and are eventually degraded by the lysosome, bafilomycin A1 treatment increased accumulation of the detergent-insoluble α-synuclein aggregates in astrocytes (Fig. 7A). Under this condition, we measured the levels of a pro-inflammatory cytokine TNFα and a chemokine CXCL1 released from astrocytes. Astrocytes exposed to neuronal cell-derived α-synuclein showed drastic inductions of TNFα and CXCL1 (Fig. 7B). Secretion of these proteins was further elevated when the cells were treated with bafilomycin A1, a situation where the cells were exposed to the same amount of extracellular α-synuclein but accumulate larger quantity of internalized α-synuclein aggregates in the cytoplasm. This suggests that the accumulation of the neuron-derived α-synuclein within astrocytes leads to the inflammatory responses. However, this does not exclude the possible extracellular action of α-synuclein in inducing astroglial inflammatory responses.

DISCUSSION

Traditionally, studies of neurodegenerative diseases have focused on the physiological and pathological abnormalities of specific neuronal cell populations. However, the role of astroglia, the most abundant cell population in the central nervous system, is increasingly evident in these diseases (42, 43). In PD and DLB, extensive α-synuclein-positive astroglial inclusion bodies have been documented in cortical and subcortical regions (4, 6, 7). In multiple system atrophy, another synucleinopathy, α-synuclein-positive inclusions, called glial cytoplasmic inclusions, are generated in oligodendrocytes (44). Astrocytes and oligodendrocytes appear to express α-synuclein protein, but the level of expression is much lower than in neurons (9, 10). The low level endogenous α-synuclein expression in glial cells has prompted a debate as to the origin of the glial α-synuclein deposition. This study demonstrates that α-synuclein aggregates released from neurons can be internalized by adjacent astrocytes, where they form glial inclusion bodies, similar to the ones found in PD, and trigger immune responses. These results support the role of neuron-derived extracellular α-synuclein in mediating pathogenic communication between neurons and astrocytes in the parenchymal microenvironment. This may also be relevant to the generation of glial cytoplasmic inclusions in multiple system atrophy.

Astrocytes participate in innate immune responses in the central nervous system and may also play a role in adaptive
immunity (45). Although evidence is emerging to support a role for astrocytic neuroinflammatory responses in neurodegenerative disease, the endogenous triggers of the disease-associated immune responses remain elusive. Our study has identified a novel pathway that links defects in a specific neuronal protein, α-synuclein, to pathological and neuroinflammatory astrocyte responses, including induction of cytokines and chemokines as well as cell adhesion molecules involved in cell migration. However, this study does not exclude the potential contribution of other proteins co-secreted with α-synuclein, although the secreted proteins from the control cells expressing LacZ gene do not show such effects. The glial pathology and associated glial responses are also typical features of “tauopathies,” including AD, progressive supranuclear palsy, corticobasal degeneration, and Pick disease (46–50). Therefore, the work presented here potentially identifies a fundamental principle of a wide spectrum of neurological diseases.

Chronic inflammation is a critical component in progression of many neurodegenerative diseases, including PD. Microglia are the major resident immune cells of the central nervous system and are responsible to a large extent for elevated inflammatory reactions in disease-inflicted brains (51, 52). Recent studies show that extracellular treatment with aggregated recombinant α-synuclein causes a variety of inflammatory responses in rodent and human microglia (15, 53–55). However, it remains to be addressed whether neuron-derived α-synuclein can cause similar microglial responses. In addition to the direct effect of α-synuclein on microglia, astrocytes may mediate microglial activation. In a transgenic mouse model of amyotrophic lateral sclerosis, astrocyte-specific deletion of the mutant superoxide dismutase 1 gene did not affect disease onset, but it significantly delayed microglial activation and late disease progression (56). This implies that astrocytes control the timing of microglial activation, leading to accelerated neuronal damage and disease progression. These results warrant future studies of the communication between astrocytes and microglia in inflammatory reactions triggered by neuron-derived α-synuclein.

In our previous study, we suggested that endocytosis of extracellular α-synuclein aggregates is part of its clearance mechanisms, based on the fact that the internalized proteins traffic along the endosomal pathway and eventually are degraded in the lysosome (35). The current study shows that astrocytes take up neuronal cell-derived α-synuclein by endocytosis and target the protein to the lysosome. This suggests that neuron-to-graft transmission of α-synuclein is a normal process whereby astroglia clear potentially harmful protein aggregates from the parenchymal microenvironment. Recent studies have shown astrocyte-mediated clearance of the Aβ protein, which implies that astrocytes serve as potential elimination sites for toxic extracellular protein aggregates (57, 58). However, in case of sustained exposure to protein conformational stresses, neuronal release of α-synuclein aggregates could persist to the extent that it exceeds the capacity of glial cell lysosomal degradation, causing the aggregates to accumulate in the glial cytoplasm. Indeed, we showed that lysosomal inhibition led to the increased accumulation of α-synuclein aggregates in astrocytes. Importantly, the glial inflammatory responses correlate with the extent of α-synuclein aggregate deposition, further emphasizing the significance of aggregate clearance in astrocytes.

Release of neuronal α-synuclein is promoted under stress conditions and can occur without apparent membrane damage; this occurs via vesicle-mediated exocytosis under the condition where membrane leakage is undetectable.3 However, high level expression of α-synuclein did cause neuronal cell death eventually, and in such a situation, the neuron-to-astroglia transmission would be more robust. Therefore, although transmission of α-synuclein can occur without apparent cell death, degenerating neurons acutely provide large amounts of α-synuclein and thereby may accelerate the transmission. Because neurons are progressively degenerating in disease-inflicted brain, we speculate that α-synuclein proteins released from degenerating neurons might play a significant role in propagation of α-synuclein pathology.

In conclusion, α-synuclein proteins released from neurons may be the source of astroglial α-synuclein pathology and an important mediator of astrogial inflammatory responses. α-Synuclein is a typical cytosolic protein, and therefore, discussions on its role in the pathogenesis of PD and other synucleinopathies have largely been limited within the neurons that highly express this protein. However, we propose an entirely novel non-cell autonomous mechanism of action for α-synuclein in disease onset and progression. The proposed model can explain some of the unexpected, yet significant experimental findings such as the protective effects of α-synuclein immunization in a transgenic mouse model (24). Our study provides the critical knowledge base for future mechanistic studies and development of immunization therapies. Recently, neuron-to-neuron transmission of α-synuclein aggregates has been demonstrated in tissue cultures and in transgenic mice with neuronal progenitor cell grafts (17). Transferred α-synuclein induces Lewy-like inclusion bodies and apoptotic changes in the recipient neurons. Therefore, neuron-derived α-synuclein, perhaps when in excess, is transferred and accumulates in neurons and glia, inducing pathological inclusion formation and degenerative changes. These data can explain the host-to-graft propagation of Lewy-like pathology in long term mesencephalic transplants in PD (18, 19) and the stereotypic topographical progression of Lewy pathology in PD suggested by Braak and colleagues (59). This non-cell autonomous transmission behavior of disease-linked aggregation-prone proteins has also been reported with polyglutamine proteins and Tau (60–62), and the templated transmission of the conformational properties has been demonstrated with recombinant Tau and α-synuclein proteins (63, 64). Therefore, increasing our knowledge of cell-to-cell transmission of α-synuclein between brain cells can provide new insights into the mechanism of disease progression and identify molecular targets for diagnosis and therapeutic intervention in PD and other synucleinopathies.

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