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γ-Synuclein: Seeding of α-Synuclein Aggregation and Transmission between Cells

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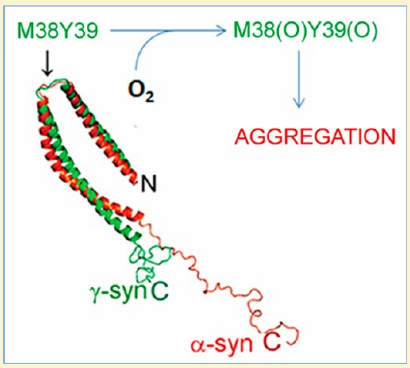
ABSTRACT: Protein misfolding and aggregation is a ubiquitous phenomenon associated with a wide range of diseases. The synuclein family comprises three small naturally unfolded proteins implicated in neurodegenerative diseases and some forms of cancer. α-Synuclein is a soluble protein that forms toxic inclusions associated with Parkinson's disease and several other synucleinopathies. However, the triggers inducing its conversion into noxious species are elusive. Here we show that another member of the family, γ-synuclein, can be easily oxidized and form annular oligomers that accumulate in cells in the form of deposits. Importantly, oxidized γ-synuclein can initiate α-synuclein aggregation. Two amino acid residues in γ-synuclein, methionine and tyrosine located in neighboring positions (Met[38] and Tyr[39]), are most easily oxidized. Their oxidation plays a key role in the ability of γ-synuclein to aggregate and seed the aggregation of α-synuclein. γ-Synuclein secreted from neuronal cells into conditioned medium in the form of exosomes can be transmitted to glial cells and cause the aggregation of intracellular proteins. Our data suggest that post-translationally modified γ-synuclein possesses prion-like properties and may induce a cascade of events leading to synucleinopathies.



Protein misfolding and aggregation is one of the most exciting new frontiers in protein chemistry and molecular medicine. These processes may be associated with a gain of toxic function and a loss of physiological function. Abnormal aggregation of neuronal proteins plays a key role in the pathogenesis of many neurodegenerative diseases (NDDs).[1−5] Parkinson's disease (PD) is characterized by the loss of the dopaminergic neurons and the deposition of α-synuclein-containing Lewy bodies.[4−6] α-Synuclein aggregation and dopamine (DA) metabolism are linked to PD pathogenesis.[7,8] Neurotransmitter DA and its derivatives promote accumulation of oligomeric or protofibril forms of α-synuclein.[9,10]

Another property of α-synuclein that is associated with its role in pathology in addition to its propensity to aggregate is its ability to be secreted.[11−15] Furthermore, the transmission of aggregated α-synuclein between cells might be considered as a mechanism of transmission of pathology.[16]

Both monomeric and oligomeric α-synuclein forms are secreted from differentiated human neuroblastoma cells and primary cortical neurons.[17] Extracellular α-synuclein aggregates are neurotoxic for any cells exposed to them. Recent data suggest that the mechanism of internalization of extracellular α-synuclein is dependent on the assembly state of the protein.[18] Transmission of α-synuclein from neurons to astroglia causes the formation of inclusion bodies and alterations in the pattern of gene expression consistent with the inflammatory response.[19] This transmission of α-synuclein might be an important mediator of pathogenic glial responses and therefore may be considered as a new therapeutic target. Exogenously added to cell culture medium, recombinant α-synuclein is internalized by the recipient cells,[18,20−22] leading to cell death.[23−25]

The interaction with recombinant α-synuclein can activate microglial cells and cause neurotoxicity.[19,25,26] In addition, α-synuclein aggregates can propagate pathology via neuron-to-neuron transmission.[27−29] The mechanism of secretion and endocytosis may play an important role in the pathogenesis of synucleinopathies, because intercellular cell-to-cell transfer of α-synuclein causes aggregation of α-synuclein in recipient neurons.[29] Thus, pathogenic actions of α-synuclein extend to the extracellular space and neighboring cells.[30]

Aggregational properties and the mechanism of secretion and endocytosis are investigated primarily for α-synuclein,[29−31] whereas much less is known about two other members of the synuclein family, β- and γ-synuclein (Figure 1A, reviewed in ref 3). γ-Synuclein, like α-synuclein, is able to form aggregates and filaments both in vitro[32] and in vivo.[33] Such γ-synuclein inclusions are found in histopathological lesions in several reports of human pathology[33−36] and in transgenic models.[37−39]

Because γ-synuclein is considered a causative factor in the development of certain neurological disorders because of its

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aberrant accumulation, these disorders may be termed γ-synucleinopathies.[33,38]

Here we show that γ-synuclein can seed α-synuclein aggregation; this seeding is especially efficient after γ-synuclein oxidation. Furthermore, γ-synuclein can be secreted from neuronal cell cultures and internalized by glial cells. These processes might have an important role in the development and spreading of synucleinopathies.

EXPERIMENTAL PROCEDURES

Antibodies (Abs) for WB and Immunofluorescence. The following Abs were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA): α-synuclein-specific antibody [sc-7011-R (C-20)] and CD63-specific Ab (H-193) (sc-15363). β-Synuclein anti-rabbit polyclonal Ab AB5068 was obtained from Chemicon (Temecula, CA). γ-Synuclein Abs used for WB were either anti-rabbit (Ab6169) or anti-mouse (1H10D2) clones from ABcam (Cambridge, MA). Mouse monoclonal Ab 2C3 against γ-synuclein (GenTex) was used for immunofluorescence. The Ser^{129}-phosphorylated α-synuclein was revealed with anti-mouse monoclonal Ab from Waco, which recognizes predominantly aggregated α-synuclein.[21] Lectin-PHA-L-conjugated by Alexa488 (Invitrogen/Molecular Probes, Eugene, OR) was used as a membrane marker and anti-mouse Ab against GM-130 [Becton Dickinson (BD), Franklin Lakes, NJ] as a Golgi marker. Secondary Abs (goat anti-mouse Alexa488 and Qdot-655 anti-rabbit) were purchased from Invitrogen/Molecular Probes. Anti-rabbit Cy3-conjugated Ab was from Jackson ImmunoResearch Lab, Inc. (West Grove, PA).

Generation of Ab to γ-Synuclein with an Oxidized Methionine 38 (Met^{38}) (21st Century Biochemicals, Marlboro, MA). Ab was raised in rabbits using as an antigen peptide Ac-TKEGV[M-O]YVGAKT-Ahx-C-amide, where [M-O] is methionine sulfoxide. The peptide was purified by high-performance liquid chromatography, and its sequence was confirmed by MS/MS Nanospray analysis. After the fourth bleeding, the Ab was affinity purified on a column containing antigenic peptide. Purified Ab was immunodepleted using an immunodepletion column with unmodified peptide without oxidized methionine (acetyl-TKEGVMYVGAKT-Ahx-C-amide). For Western blotting (WB), the Ab was used at a 1:500 dilution.

Analysis of α-, β-, and γ-Synucleins. *Colloidal Coomassie Brilliant Blue Staining.* The protein samples were separated in a 12% gel and fixed in 12.5% TCA for 1 h. The gel was stained with 0.1% G-250 brilliant blue in 20% methanol containing 10% $(NH_4)_2SO_4$ and 2% H_3PO_4 overnight at RT and washed with H_2O.

Aggregation Assays. In Vitro Assay. Recombinant α-, β-, and γ-synucleins were from ABcam (α-synuclein ab48842, β-synuclein ab48853, and γ-synuclein ab48712, respectively). The proteins were diluted by PBS to 0.5 μM, mixed with a 20-fold excess of DA, and incubated overnight at 37 °C. The samples were boiled for 3 min in a sample buffer (SB) with 5% β-mercaptoethanol. After the treatment, we assessed the formation of SDS-resistant protein aggregates by electrophoresis in a 12% or 4 to 15% PAAG.

Assay of Cell Extracts. A7 cells were split in a 96-well plate in the regular medium. The next day, γ-synuclein preincubated with DA or PBS buffer was added to the cells. The cells were washed with PBS after different periods of incubation (0, 6, 24, and 48 h) and suspended in 2× SB. The samples were boiled and analyzed in a 4 to 15% PAAG in the presence of SDS.

Incubation with DA or L-DOPA. α-, β-, and γ-synucleins were incubated overnight with a 20-fold molar excess of DA in PBS. Because of the low stability of DA in experiments with longer periods of incubation, it was replaced with a similar concentration of a more stable DA precursor, 3,4-dihydroxyphenyl-L-alanine (L-DOPA).

Cell Cultures. The immortalized primary culture of rat optic nerve A7 astrocytes was a generous gift from H. Geller (National Institutes of Health, Bethesda, MD).[40] The culture was immortalized with murine leukemia virus psi-2, SV-40-6, which is defective in assembly and contains the SV-40 large T antigen and neomycin-resistance genes, as described previously.[40] This stable immortalized clonal cell line expressed nuclear SV-40 large T cells and the astrocyte-specific marker glial fibrillary acidic protein (GFAP). The cells were grown as described previously.[41−43]

Human neuroblastoma cell line SH-SY5Y was purchased from ATCC (catalog no. CRL-2266) and cultivated in DMEM/F-12 medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. Astrocytoma grade IV/high-grade astrocytoma (U-87 MG) (ATCC, catalog no. HTB-14) was cultivated in Eagle's Minimal Essential Medium with Earl's BSS and 2 mM glutamine (EMEM) adjusted to contain 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 1.5 g/L sodium bicarbonate (90%) and fetal bovine serum (10%). Other details are described by the manufacturer and in our previous publication.[42]

Immunofluorescent Staining. *SH-SY5Y Cells.* They were split on coverslips covered with poly-D-lysine. The cells were washed with cold PBS and stained with PHA-L conjugated with Alexa488 for 15 min at 4 °C before being fixed (4% PFA for 15 min at RT). After being fixed, the cells were washed and blocked in 3% BSA, 3% NGS, and 0.1% Triton X-100 in PBS for 2 h at RT. The coverslips were incubated with the primary Ab overnight at 4 °C. After being stained with the secondary Ab for 1 h at RT, the coverslips were put in a mounting solution containing DAPI-Vectashield (Vector Laboratories, Burlingame, CA). Other details are described in ref 44.

U-87 MG Cells. They were split on the coverslips in a 12-well plate in the regular medium. The next day, the medium was replaced for the CMs from the stable clones produced in SH-SY5Y cells. The cells were grown for 7 or 12 days at 37 °C in the presence of 5% CO_2. Before being fixed, the cells were washed with warm PBS, containing Ca^{2+} and Mg^{2+}, and then fixed with 4% PFA for 15 min at RT. The cells were washed with PBS, permeabilized with 0.3% Triton X-100 (15 min at RT), and blocked in a solution containing 3% BSA, 3% NGS, and 0.1% Triton X-100 in PBS for 2 h at RT. The coverslips were placed in the primary antibody overnight at 4 °C. After being stained with the secondary antibody for 1 h at RT, the coverslips were placed in a mounting solution containing DAPI-Vectashield (Vector Laboratories). Nonfixed cells were washed with PBS and stained with PHA-L conjugated with Alexa488 for 2 h at RT before being fixed with 4% PFA for 15 min at RT.

Confocal Microscopy. The images of U-87 cells were taken with a Nikon TE 2000 Inverted 3 Laser confocal microscope driven by Nikon EZ-C1 3.91 software. The following lasers were used: 488, 543, and 633 nm. Nikon EZ-C1Free Viewer software was used for the reconstruction of orthogonal projections.

Expression of γ-Synuclein in SH-SY5Y Cells. For the generation of γ-synuclein-overexpressing clones, pcINeo plasmid-containing human γ-synuclein cDNA was transfected into SH-SY5Y cells using Lipofectamine 2000 (Invitrogen/Molecular Probes). The clones resistant to antibiotic G-418 (300 μg/mL) were selected. For the generation of γ-synuclein and mutant γ-synuclein fused with GFP clones, the pEGFP-N1 vector was used as a template for amplification with primers containing restriction site recognition sequences for cloning (Clontech, Mountain View, CA). The following primers were used: 5′-ATC TCG AGA TGG ATG TCT TCA-3′ (forward) (with the *Xho*I recognition site) and 5′-TTA AGC TTG TCT CCC CCA CTC-3′ (reverse) (with the *Hin*dIII site). Amplification was conducted with a kit for GC-rich sequences (Roche), and the generated polymerase chain reaction product was cloned into the *Xho*I and *Hin*dIII sites of the pEGFP-N1 vector. γ-Synuclein-GFP mutant clones were generated by the same procedure, the exception being that pTrc-His6-γ-synuclein constructs that already had mutations in cDNAs were used for the expression of proteins in *Escherichia coli*.

Overexpression of wt and A53T Mutant α-Synuclein. SH-SY5Y cells overexpressing wild-type (wt) and A53T mutant α-synuclein were generated using lentivirus constructs as described previously[27] (generous gift of E. Masliah). In one set of experiments, the stable clones overexpressing wt-γ-synuclein fused with GFP were infected with the lentivirus construct carrying wt-α-synuclein. The cells were grown for 48 h in the regular medium containing 300 μg/mL antibiotic G-418. Extracts were analyzed by WB with anti-rabbit Ab C-20 (Santa Cruz Biotechnology, Inc.) specific for α-synuclein.

Delivery of γ-Synuclein into the SH-SY5Y Cell Line by BioPorter. Cells were split in a 24-well plate in the regular medium. γ-Synuclein was diluted with PBS to 0.1 mg/mL, treated by 1 mM DA, and incubated overnight at 37 °C. Protein samples were adjusted to 40 μL by PBS and transferred to tubes with lyophilized BioPorter (Sigma). Subsequent procedures were conducted as recommended by the manufacturer. Before the mixtures with BioPorter were added to the cells, the regular medium was replaced with SFM (Invitrogen, Carlsbad, CA). The cells were grown for 24 h; the serum was added to a final concentration of 10%, and the growth was continued for an additional 24 h. FITC-IgG protein (Sigma) was used as a control.

Secretion of γ-Synuclein from SH-SY5Y Cells. SH-SY5Y stable clones overexpressing γ-synuclein or γ-synuclein-pGFP were grown for 24 h in the presence of 10% FBS, and the medium was replaced with SFM (Gibco/Life Technologies, Grand Island, NY). After 48 h, CM was collected and added to astrocytoma U-87 cells. The U-87 cells were grown for 72 h, after which the CM was spun and concentrated six times using an Amicon device. The cells were washed and suspended in buffer, and CE and CM were analyzed by WB in a 12% PAAG. γ-Synuclein-specific Ab 1H10D2 was used as the primary Ab.

Isolation of Exosomes by High-Speed Centrifugation. The stable SH-SY5Y clones overexpressing γ-synuclein or γ-synuclein-GFP were split in a six-well plate in the regular medium. One day after the cells had reached 75−80% of the density, the medium was replaced with SFM (Invitrogen). After 48 h, the medium was collected into the sterile Eppendorf tubes and centrifuged for 15 min at 2000*g*. The supernatant was centrifuged for 20 min at 12000*g*; the pellet was discarded, and the supernatant was subjected to ultracentrifugation (125000*g* for 70 min at 4 °C). The pellets were washed with cold PBS and suspended in 10 μL of hot BD buffer, and then an equal volume of 2× SB containing β-mercaptoethanol (ME) was added. The samples were boiled and loaded onto a 12% PAAG. The cells from the same wells were also washed with cold PBS and suspended in hot BD buffer, boiled, sonicated, and measured for their protein content using the BCA-1 protein assay kit (Sigma, St. Louis, MO).

Isolation of Exosomes Using ExoQuick Precipitation. SH-SY5Y cells overexpressing γ-synuclein were grown in the regular medium for 24 h; the medium was replaced with SFM, and growth was continued for 48 h. CM was collected and spun (3000*g* for 10 min) to remove cells and cellular debris. Exosomes were precipitated from CM by ExoQuick (System Biosciences, Mountain View, CA) as follows. CM was incubated overnight at 40 °C with ExoQuick in a 1:2 ratio. After 24 h, the mixture was centrifuged at 13000*g* for 30 min and both the supernatant and the pellet were analyzed by WB.

Treatment with Inhibitors of Exosome Biogenesis. Two hours before the media was replaced with SFM, methyl-β-cyclodextrin (Sigma) was added directly to the medium to a final concentration of 25 μg/mL. A cell-permeable inhibitor of neutral sphingomyelinase, GW4869 (Sigma), was dissolved in DMSO and added for 48 h to SFM to a final concentration of 7.5 μM. The same amount of DMSO was added to control wells.

Western Blotting. The protein samples were analyzed by WB in a 12% PAAG in the presence of SDS as described previously;[41,42] 30 μg of total protein was loaded on each well. After electrophoresis, proteins were transferred onto an Immobilon-FL transfer membrane with a pore size of 0.45 μm (Millipore, Chelmsford, MA). Nonspecific binding sites were blocked by immersing the membrane in Tris-buffered saline with 0.05% Tween 20 (TBST) and 10% nonfat dry milk for 1 h at room temperature on an orbital shaker. For quantitative imaging of films, Kodak Image Station 440 CF (Eastman Kodak Co., Rochester, NY) and Kodak Digital Science Image Analysis were used. Other details have been described previously.[41−44]

In Vitro Mutagenesis. Met[38] and Tyr[39] in recombinant proteins were substituted for Ala using a Stratagene/Agilent Technologies, Inc. (Santa Clara, CA), Quick Change Site-Directed Mutagenesis Kit (catalog no. 200518) following the procedures recommended by the manufacturer. For the replacement of Met[38] with Ala, oligonucleotide 5′-CCA AGG AGG GGG TCG CGT ATG TGG GAG CCA-3′ was used; for the replacement of Tyr[39] with Ala, we used oligonucleotide 5′-AAG ACC AAG GAG GGG GTC ATG GCA GTG GGA GCC AAG ACC-3′. The complement oligonucleotide was 5′-GGT CTT GGC TCC CAC TGC CAT GAC CCC CTC CTT GGT CTT-3′. For the replacement of both Met[38] and Tyr[39] with Ala, we used oligonucleotide 5′-AAG ACC AAG GAG GGG GTC GCG GCA GTG GGA GCC AAG ACC-3′ and as a complement oligonucleotide 5′-GGT CTT GGC TCC CAC TGC CGC GAC CCC CTC CTT GGT CTT-3′. Other details of the procedure have been described previously.[43] cDNA pTrc-His-γ-synuclein was used as a template.

Generation of Recombinant γ-Synuclein. Recombinant proteins were expressed in *E. coli* using cDNA pTrc-His-γ-synuclein in the presence of 1 mM IPTG as an inducer of expression. Proteins were purified using a ProBond (Invitrogen/Life Technologies Corp., Grand Island, NY) column following the instructions for ProBond column users. Purified proteins were at least 98% pure.

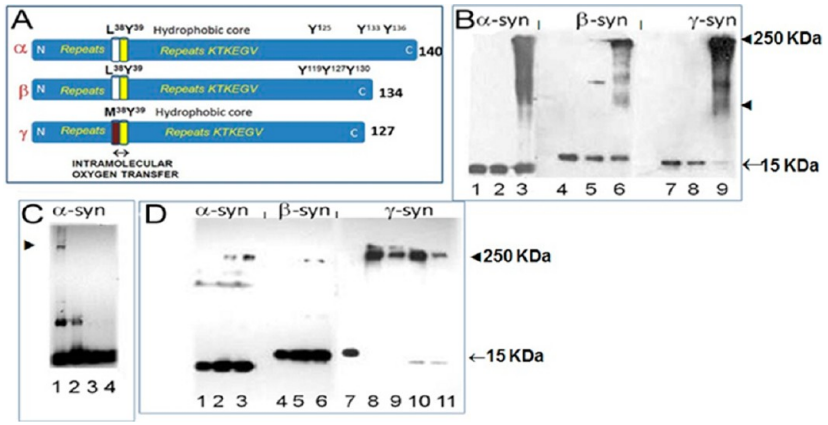

Figure 1. Aggregation of α-, β-, and γ-synucleins in vitro and in cell cultures. (A) Schematic drawing of α-, β-, and γ-synucleins. (B) Synuclein aggregation in vitro. Samples 1, 4, and 7 were incubated at 4 °C; samples 2, 5, and 8 at 37 °C; and samples 3, 6, and 9 at 37 °C with 1 mM DA. WB was conducted with Ab to α-synuclein (lanes 1−3), β-synuclein (lanes 4−6), and γ-synuclein (lanes 7−9). Significant amounts of aggregated α- and γ-synucleins were formed after overnight incubation of proteins at 37 °C in the presence of DA (lanes 3 and 9 between arrowheads). α- and β-synuclein monomers (arrow) were converted to aggregates only partially (lanes 3 and 6), whereas γ-synuclein was almost completely turned into aggregates (lane 9). (C) Oxidized wt-γ-synuclein induces aggregation of α-synuclein. γ-Synuclein was oxidized, separated from DA, and incubated overnight with α-synuclein. The samples were subjected to WB with α-synuclein-specific Ab: lane 1, α-synuclein incubated with oxidized γ-synuclein in a 5:1 ratio; lane 2, same as lane 1, but with a 20:1 ratio; lane 3, α-synuclein incubated with untreated γ-synuclein in a 5:1 ratio; lane 4, same as lane 3, but with a 20:1 ratio. The formation of both α-synuclein dimers and aggregates (~250−300 kDa) was observed only when α-synuclein was incubated with oxidized γ-synuclein in a 5:1 ratio (lane 1, arrowhead). (D) Oxidized γ-synuclein forms a heteromeric complex in vitro with α-synuclein but not β-synuclein. γ-Synuclein was treated with DA and incubated with α-synuclein (lanes 2, 3, 8, and 9) or β-synucleins (lanes 5, 6, 10, and 11); DA was eliminated by dialysis in samples 2, 5, 8, and 10. WB was performed using Ab to α-synuclein (lanes 1−3), β-synuclein (lanes 4−6), and γ-synuclein (lanes 7−11). Lane 7 contained untreated γ-synuclein. γ-Synuclein nucleates aggregation of α-synuclein, but not β-synuclein. High-molecular mass aggregates (~250−300 kDa, arrowhead) contain both α-synuclein (lanes 2 and 3) and γ-synuclein (lanes 8 and 9).

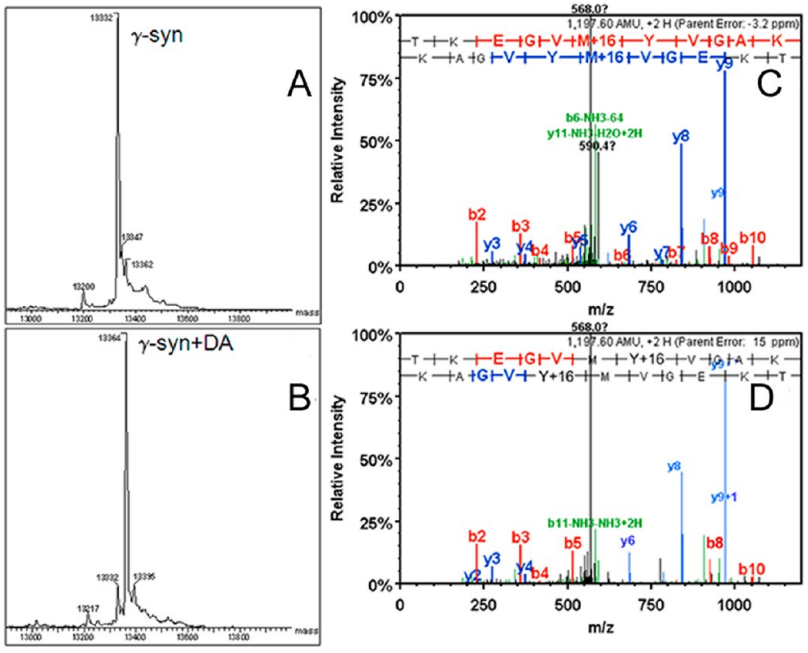

Figure 2. Mass spectrometry analysis of γ-synuclein. (A and B) Q-TOF spectra of the whole (undigested) γ-synuclein incubated with either buffer (A) or DA (B). An increase of 32 Da was found for the DA-treated protein sample. (C and D) Representative capLC-LTQ-FT-MS/MS spectra demonstrating the presence of oxidized (mass increase of 16 Da) Met^{38} (C) or Tyr^{39} (D) in the γ-synuclein tryptic peptide sequence TKEGVM^{38}Y^{39}VGAK after DA treatment.

TEM of γ-Synuclein Aggregates. γ-Synuclein was incubated with or without DA. After being incubated overnight at 37 °C, the samples were applied to 300 mesh Formvar/carbon-coated grids and negatively stained with 1% uranyl acetate. A JEOL 100CXII TEM instrument was used to examine the samples. Images were photographed at a magnification of 36000× on Kodak electron image film. Negatives were developed and scanned into TIF files using an Epson Perfection 4990 photo scanner.

Mass Spectrometry. The mass spectra of the undigested protein were recorded using a Q-TOF-2 (Micromass Ltd., Manchester, U.K.) mass spectrometer. CapLC-LTQ-FT-MS/

MS spectra for in-solution or in-gel protein digests were recorded on an LTQ-FT instrument (ThermoElectron, San Jose, CA) as described previously.[45] Additionally, raw data were examined manually using Mass Matrix (http://www.massmatrix.net).

Inhibition of Proteasome Activity. SH-SY5Y cells were grown for 24 h in the regular medium with subsequent replacement of the medium with SFM. Cells were treated with proteasome inhibitor MG132 (10 μM for 24 h). CM was harvested, centrifuged (10 min at 3000g), and concentrated 10 times using the Amicon device with membrane-retaining proteins with a molecular mass of >10 kDa.

Statistical Analysis. At least three sets of WB were performed for each experiment. A Student's t test was used to assess significant differences between groups. The effect of inhibitors was analyzed by ANOVA.

RESULTS

Aggregation of Synucleins in Vitro. Incubation of synucleins at 37 °C is accompanied by a slow oligomerization and aggregation. DA significantly accelerates the level of synuclein oligomerization, producing protein complexes with molecular masses of ~250 kDa (in Figure 1B, aggregates are seen in lanes 3, 6, and 9 between arrowheads). Importantly, γ-synuclein most easily forms high-molecular mass aggregates compared to two other members of the family. Almost all γ-synuclein monomer (arrow) was converted to oligomeric form (Figure 1B, lane 9 between arrowheads), whereas the conversion of α- and β-synuclein monomers to aggregated species was only partial (Figure 1B, lanes 3 and 6, arrow). Furthermore, oxidation of γ-synuclein not only causes its aggregation but also induces its ability to seed the aggregation of α-synuclein (Figure 1C, lane 1, arrowhead; Figure 1D, lanes 2, 3, 8, and 9). Aggregation of β-synuclein was not affected (Figure 1D, lanes 5 and 6). At a higher α-synuclein:γ-synuclein ratio (20:1) (Figure 1C, lane 2) or after incubation of α-synuclein with untreated γ-synuclein (Figure 1C, lanes 3 and 4), α-synuclein aggregates were not found. Because γ-synuclein-induced α-synuclein aggregates (~250 kDa) are γ-synuclein immunopositive (Figure 1D, lanes 9−11), they can be considered as heterooligomers containing both these proteins.

Analysis of DA-Treated γ-Synuclein by Mass Spectrometry. We then sought to determine whether the increased aggregation propensity of γ-synuclein after DA treatment is due to a chemical modification of the protein. Analysis of mass spectra of the whole (undigested) protein revealed that after DA treatment most of the protein (~90%) was converted to the doubly oxidized isoform (addition of 32 Da to the original mass of 13332 Da). Approximately 10% of the sample was represented by an intact protein, while no monooxidized isoform was found (Figure 2A,B). Tandem MS analysis of γ-synuclein tryptic digests after DA treatment showed the presence of oxidized Met^{38} and Tyr^{39} (panels C and D of Figure 2, respectively). No other oxidation modifications have been detected at ~70% sequence coverage. The MS/MS spectra for the two adjacent oxidation sites are very similar, have exactly the same ion precursor mass, and can be distinguished only through a difference in y5 (537.3 vs 553.3) and b4 (415.2 vs 399.2) fragment ion m/z values for Met^{38}(O) and Tyr^{39}(O).

Role of Met^{38} and Tyr^{39} in the Propensity of γ-Synuclein To Aggregate. We then asked whether the oxidation of Met^{38} and Tyr^{39} might play a role in the susceptibility of γ-synuclein to aggregation in vitro. For this purpose, we replaced these two residues with alanine and compared the propensity of wt and mutant forms of γ-synuclein to aggregate. After incubation with DA, wt-γ-synuclein forms high-molecular mass aggregates that can be detected by Coomassie staining (Figure 3A, lane 3, arrowhead). Similar

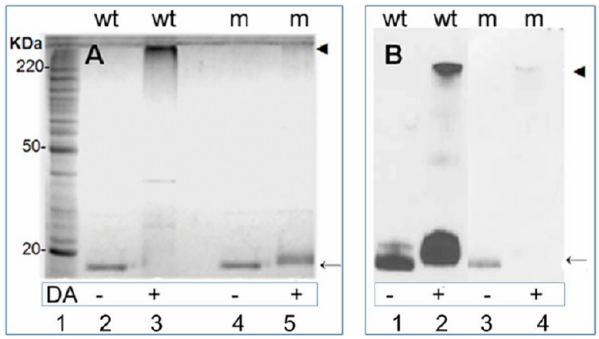

Figure 3. Role of Met^{38} in the propensity of γ-synuclein to aggregate. (A) Substitution of Met^{38} reduces the propensity of γ-synuclein to aggregate. The recombinant protein with Met^{38} substituted for Ala was incubated with DA (lanes 3 and 5) or buffer (lanes 2 and 4). After PAGE, the gel was stained with Colloidal Coomassie Brilliant Blue. Almost all wt-γ-synuclein forms high-molecular mass aggregates (lane 3, arrowhead), whereas a mutant with the Met^{38} →Ala substitution remains basically monomeric (lanes 4 and 5, arrow): lane 1, molecular mass markers; lane 2, wt-γ-synuclein incubated without DA; lane 3, wt-γ-synuclein incubated with DA; lane 4, γ-synuclein with the Met^{38} → Ala substitution incubated with buffer; lane 5, γ-synuclein with the Met^{38} → Ala substitution incubated with DA. (B) The Ab raised to γ-synuclein with oxidized Met^{38} reveals a high-molecular mass form of γ-synuclein (lane 2, arrowhead). Such aggregates are absent in untreated γ-synuclein with the Met^{38} → Ala substitution (lane 3) and in nonoxidized wt-γ-synuclein (lane 1). wt, wild-type γ-synuclein; m, Met^{38} → Ala mutant; +, incubation with DA; −, incubation with buffer.

size aggregates were revealed by WB (Figure 1B, lane 9); however, only the most abundant forms of aggregates were revealed by Coomassie staining because of the lower sensitivity of this method of detection. The substitution of Met^{38} with Ala reduced the level of aggregation of the protein (lane 4, untreated; lane 5, DA-treated M^{38}A γ-synuclein), the majority of which remained in the form of a monomer (Figure 3A, arrow). Similar results were obtained for γ-synuclein in which Tyr^{39} had been substituted with Ala (not shown). Thus, the propensity for γ-synuclein to aggregate under oxidative conditions in vitro depends on the presence of Met^{38} and Tyr^{39} in adjacent positions. The role of these amino acids in the structural organization and fibrillation of α-synuclein[46,47] and in model peptides[48] has been demonstrated previously. The presence of the Met^{38}-oxidized form of γ-synuclein in the fraction of aggregated protein was confirmed by the use of Ab raised to γ-synuclein with oxidized Met^{38} (Figure 3B, lane 2, arrowhead). This Ab reveals only minor amounts of aggregates in the recombinant γ-synuclein with the Met^{38} → Ala substitution (Figure 3B, lane 4).

Analysis of Synuclein Oligomers by TEM. According to TEM, γ-synuclein oligomers generated after oxidative treatment have a ring- or horseshoelike shape with a size of 30−50 nm (Figure 4A,B,D) and look like the annular oligomers described previously for α-synuclein.[49,50] Similar but less numerous annular oligomers are formed upon incubation of γ-synuclein in

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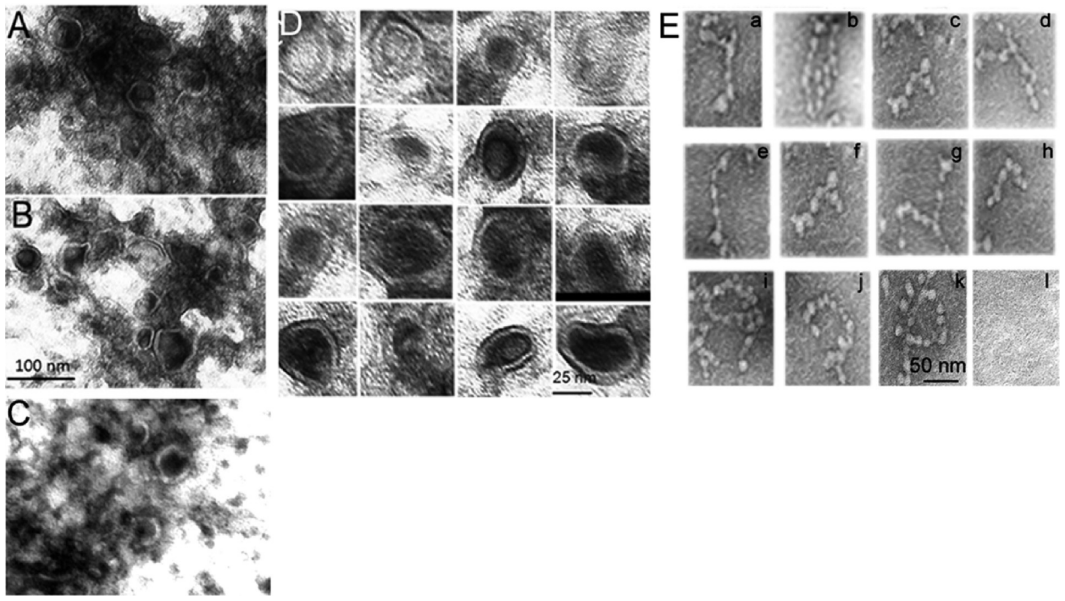

Figure 4. Analysis of α- and γ-synuclein aggregates by TEM. γ-Synuclein was incubated overnight at 37 °C with DA (A and B) or without DA (C) as described in the legend of Figure 1. The samples were applied to 300 mesh Formvar/carbon-coated grids and negatively stained with 1% uranyl acetate. (A and B) Random fields of γ-synuclein incubated with DA. (C) γ-Synuclein incubated without DA. (D) Selected round aggregates. (E) TEM micrographs illustrating α-synuclein prefibrillar oligomers formed in the presence of oxidized γ-synuclein (5:1 ratio). The oligomers have either a rodlike (a–h) or an annular (i–k) shape (~200 nm length for rodlike and ~70 nm diameter for annular oligomers). (l) α-Synuclein incubated without γ-synuclein. Met^{38} → Ala substitution.

the absence of DA (Figure 4C). α-Synuclein aggregates generated in the presence of oxidized γ-synuclein have either a rodlike (Figure 4E, a–h) or an annular (Figure 4E, i–k) shape (~200 nm length for rodlike and ~70 nm diameter for annular oligomers).

γ-Synuclein Aggregation in Neuronal Cells and Astrocytes. Next we examined localization and aggregation of oxidized γ-synuclein in different types of cultured cells. According to immunofluorescence microscopy, oxidized γ-synuclein has a perinuclear punctuate appearance and may accumulate in neuronal SH-SY5Y cells in the form of intracellular inclusions (Figure 5A, panels b and d), whereas monomeric γ-synuclein is located predominantly in the cytoplasm and does not form deposits (Figure 5A, panels a and c). Addition of the oxidized oligomeric γ-synuclein to astrocytes causes its internalization and accumulation inside the cells in its aggregated form (Figure 5B, lane 6) with a molecular mass of ~250 kDa.

Then we sought to determine whether the induction of α-synuclein aggregation by γ-synuclein in vitro might occur in cells. To reveal whether γ-synuclein affects α-synuclein aggregation in neuronal cells, we used two approaches for the delivery of γ-synuclein into SH-SY5Y cells: (1) overexpression using the pEGFP-N1 plasmid containing human wild-type and mutant (Met^{38} → Ala) γ-synuclein cDNA (Figure 5C,E) and (2) protein delivery agent BioPorter (Figure 5D,F). Aggregated α-synuclein was identified with the Ab against α-synuclein phosphorylated at Ser^{129} [anti-α-synuclein (pSer^{129}) rabbit pAb], because α-synuclein accumulates in cell cultures in hyperphosphorylated states recapitulating key features of intracellular inclusions in PD.[21] As shown in lane 3 of Figure 5C and in Figure 5E, the amount of aggregated α-synuclein (molecular mass of ~200 kDa) was significantly augmented (5.6-fold increase; n = 3) when the cells were transfected with wt-γ-synuclein, while mutant γ-synuclein (Met^{38} → Ala substitution, lane 2) caused a less significant increase (3.6-fold; n = 3). A similar effect was observed when either intact γ-synuclein or DA-treated γ-synuclein was delivered by BioPorter (lane 2 or 3 of Figure 5D, respectively, and Figure 5F). Thus, γ-synuclein delivered either by transfection or by protein delivery agent enhanced the aggregation of α-synuclein in neuronal cells.

Secretion and Internalization of γ-Synuclein. According to the literature data, α-synuclein can be secreted from cells in the form of exosomes.[30] We asked whether secretion and internalization of γ-synuclein occur through a similar mechanism. To answer this question, we first overexpressed γ-synuclein in the neuronal SH-SY5Y cell culture with and without the GFP tag and analyzed the conditioned medium (CM) and cell extracts (CE) by WB. As shown in Figure 6A, both monomeric γ-synuclein and the γ-synuclein-GFP fusion protein are secreted into CM. γ-Synuclein expressed without the GFP tag is secreted predominantly as a monomer (Figure 6A, lane 4) with a size corresponding to the size of the intracellular protein (Figure 6A, lane 1). Addition of proteasome inhibitor MG132 does not affect the size of the secreted protein (not shown). γ-Synuclein fused to GFP migrates in a PAAG according to the sum of the molecular masses of two proteins (~42 kDa) as seen in lanes 2, 3, 5, and 6.

We then asked whether γ-synuclein secreted from a neuronal culture can be internalized by astrocytoma cells. SH-SY5Y stable clones overexpressing either γ-synuclein or γ-synuclein-pGFP were grown in SFM for 48 h; CM was collected, filtered through a 0.2 μm filter, and added to astrocytoma U-87 cells, which were split in the regular medium a day before. Figure 6B shows the uptake of extracellular γ-synuclein by astrocytoma cells. The majority of γ-synuclein in astrocytoma CE is present as low-molecular mass aggregates (35–60 kDa, lanes 7 and 8), whereas in CM, higher-molecular mass aggregates prevail (40–

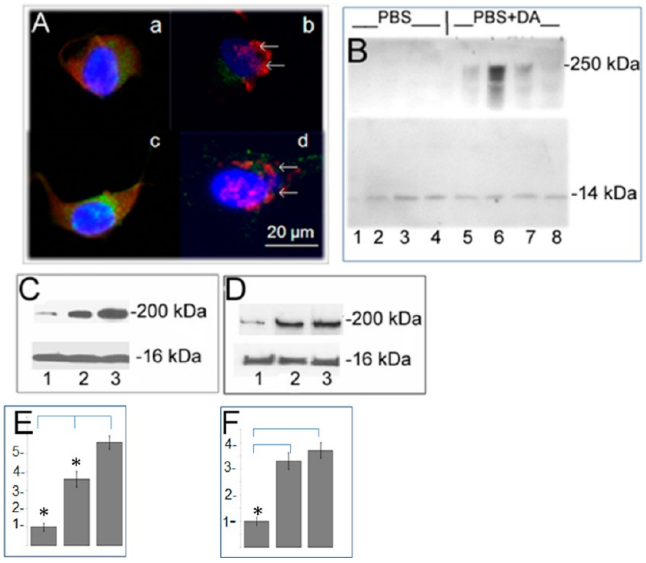

Figure 5. Internalization of γ-synucleins in glial and neuronal cells. (A) Immunofluorescent staining of neuroblastoma SH-SY5Y cells revealed the localization of monomeric and aggregated forms of γ-synuclein (red). Oxidized (b and d) and nonoxidized (a and c) forms of γ-synuclein were delivered by a cationic liposome reagent (BioPorter, Sigma). Conjugated PHA-L (Molecular Probes) labeled with Alexa488 was used as a plasma membrane marker (green, a and c). The Ab against GM-130 (Becton Dickinson) was used as a Golgi marker (green, b and d). DAPI denotes blue staining. Only oxidized γ-synuclein forms intracellular deposits (red, b and d, arrows), which are colocalized neither with the plasma membrane nor with Golgi markers. The data shown are representative of four independent experiments with similar results. (B) Addition of the aggregated γ-synuclein to A7 astrocytes causes its intracellular accumulation. γ-Synuclein was incubated overnight in PBS buffer (lanes 1−4) or in PBS with DA (lanes 5−8) and then added to cultured A7 cells. Cell extracts were subjected to PAGE in a gradient 4 to 15% gel and analyzed by WB with γ-synuclein Ab. Cells were incubated with γ-synuclein as follows: lanes 1 and 5, no incubation; lanes 2 and 6, incubation for 6 h; lanes 3 and 7, incubation for 24 h; lanes 4 and 8, incubation for 48 h. Intracellular γ-synuclein aggregates accumulated after a 6 h incubation. (C) Overexpressed γ-synuclein enhances α-synuclein aggregation in cultured SH-SY5Y cells infected with a lentivirus expressing α-synuclein: lane 1, control clone transfected with an empty vector; lanes 2 and 3, stable clones overexpressing mutant Met^{38} → Ala γ-synuclein-GFP (lane 2) and wt-γ-synuclein-GFP (lane 3). The blot was probed with the Ab specific to α-synuclein (bottom), stripped, and probed with the Ab to Ser^{129}-phosphorylated α-synuclein (Wako) that recognizes predominantly aggregated α-synuclein^{21} (top). In the presence of γ-synuclein, the amount of aggregated α-synuclein (molecular mass of ∼200 kDa) was increased 3.6 times by Met^{38} → Ala γ-synuclein-GFP (lane 2) and 5.6 times by wt-γ-synuclein (lane 3) (n = 3). (D) γ-Synuclein delivered by BioPorter enhances α-synuclein aggregation: lane 1, FITC-IgG delivered as a control; lane 2, intact γ-synuclein; lane 3, DA-treated γ-synuclein delivered with BioPorter. The Western blot was probed with the α-synuclein Ab for detection of monomeric α-synuclein (bottom), stripped, and reprobed with the Ab specific to Ser^{129}-phosphorylated α-synuclein. Both intact (lane 2) and oxidized (lane 3) γ-synuclein delivered by a protein delivery agent increased the level of α-synuclein aggregation (3.1 and 3.4 times, respectively) compared to control (lane 1) (n = 3). (E and F) Extents of induction of aggregated α-synuclein levels vs control (without γ-synuclein) presented as a bar graph corresponding to panels C and D, respectively, were calculated on the basis of densities of bands from three independent experiments. Asterisks show a statistically significant difference (p < 0.05).

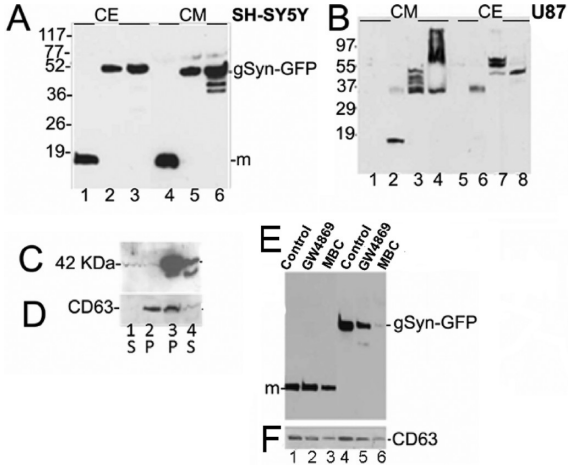

Figure 6. Secretion of γ-synuclein from neuronal cells in the form of exosomes and its transmission into astrocytes. (A) Three types of γ-synuclein overexpressing stable SH-SY5Y clones were generated. In clone 1, γ-synuclein was expressed in the pcINeo vector (lane 1); in clone 2, γ-synuclein was fused with GFP (lane 2), and in clone 3, mutant γ-synuclein with the Met^{38} → Ala substitution was fused with GFP (lane 3). Lane 4 contained CM from cells overexpressing the γ-synuclein monomer. Lane 5 contained CM from cells overexpressing γ-synuclein-GFP. Lane 6 contained CM from cells overexpressing mutant γ-synuclein-GFP. The Western blot was probed with the γ-synuclein-specific Ab. The molecular masses of marker proteins are shown at the left. (B) γ-Synuclein in astrocytoma U-87 cells and CM. CM medium from SH-SY5Y cells overexpressing γ-synuclein was added to U-87 cells. After the cells had been grown for 72 h, the CE and CM were analyzed by WB. Lanes 1−4 contained CM; lanes 5−8 contained the corresponding CE: lanes 1 and 5, U-87 control samples incubated with fresh SFM; lanes 2 and 6, samples from γ-synuclein stable clones overexpressing nonfused γ-synuclein; lanes 3 and 7, samples from the γ-synuclein-pGFP clone; lanes 4 and 8, samples from clones expressing mutant (Met^{38} → Ala) γ-synuclein-pGFP. (C) Exosome samples were precipitated with ExoQuick and subjected to electrophoresis in a 12% PAAG. γ-Synuclein fused with GFP is present in the exosomal pellet (P) precipitated from CM (lane 3) as a protein with a molecular mass of ∼42 kDa that corresponds to the sum of the molecular masses of two proteins. In the CM supernatant, this band is less abundant (C, lane 4). (D) The blot was stripped and immunoblotted with the Ab to exosome marker CD63 (Santa Cruz Biotechnology, Inc., 1:100 dilution): lanes 1 and 2, CM from control SH-SY5Y cells; lanes 3 and 4, CM from cells overexpressing γ-synuclein-pGFP; lanes 1 and 4, supernatant; lanes 2 and 3, pellets after the precipitation of exosomes. (E) Inhibitors of exosome biogenesis GW4869 and MBC reduce the level of secretion of monomeric γ-synuclein (m) and γ-synuclein fused to GFP (g-Syn-GFP) from SH-SY5Y cells: lane 1, control sample for monomeric γ-synuclein incubated without inhibitors; lane 4, control sample for the γ-synuclein-GFP-fused protein incubated without inhibitors. GW4869 caused a 58 ± 5% inhibition (n = 3) of γ-synuclein-GFP secretion (in panel E, compare lanes 4 and 5). MBC caused a significant inhibition (92 ± 4%; n = 3) of secretion of the fused protein (in panel E, compare lanes 4 and 6). Inhibition of the secretion of monomeric γ-synuclein by both inhibitors was less substantial compared to the inhibition of the fused protein. MBC reduced the level of secretion of monomeric (m) γ-synuclein by 34 ± 6% (n = 3; compare lanes 1 and 3). No statistically significant inhibition was found for GW4869 (E, lane 2). (F) The amount of secreted exosome marker CD63 was reduced in the presence of both inhibitors. The average reduction in the level of CD63 by GW4869 is 46.2 ± 6% (combined lanes 2 and 5 compared to combined lanes 1 and 4; n = 3); the average reduction by MBC was 73.6 ± 4% (combined lanes 3 and 6 compared to combined lanes 1 and 4; n = 3).

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200 kDa, lanes 3 and 4). Thus, γ-synuclein can be internalized by astrocytoma cells. Interestingly, nonfused γ-synuclein is present mainly as a monomer and a small fraction as a dimer in CM (Figure 6B, lane 2). However, in astrocytoma cells, the CE main band is a dimer and only traces of monomeric γ-synuclein are present (Figure 6B, lane 6).

Then we determined whether exosomes are implicated in γ-synuclein secretion. For this purpose, we used two methods of exosome isolation: (a) a polymer-based method of exosome precipitation with ExoQuick and (b) a differential centrifugation described previously.[30]

First, we precipitated exosomes with ExoQuick and analyzed the presence of γ-synuclein in the exosome pellet (P) with the Ab to γ-synuclein (Figure 6C) and exosomal marker CD63 (Figure 6D). γ-Synuclein fused with GFP is present in the exosomal pellet precipitated from CM (Figure 6C, lane 3) and in a considerably smaller amount in the CM supernatant (S) (Figure 6C, lane 4). γ-Synuclein-GFP migrates in a PAAG according to the sum of the molecular masses of two proteins (42 kDa). A negligible amount of this protein was found in control SH-SY5Y cells that did not overexpress γ-synuclein (Figure 6C, lanes 1 and 2). When the blot was stripped and incubated with the Ab to exosome marker CD63, the majority of the antigen was present in the pellet (Figure 6D, lanes 2 and 3), but not in the supernatant (Figure 6D, lane 1 and 4). Nonfused γ-synuclein also was localized in the exosome pellet as a protein with a molecular mass of ∼14 kDa (not shown).

Effect of Methyl-β-cyclodextrin (MBC) and GW4869 on the Secretion of γ-Synuclein. Next we sought to determine how inhibitors of exosome biogenesis affect secretion of the monomeric γ-synuclein (designated with an "m" in Figure 6E) and γ-synuclein fused to GFP (gSyn-GFP in Figure 6E). We assessed the effect of two substances that inhibit the formation of exosome components, i.e., cholesterol and ceramide. MBC is a cholesterol-depleting drug, while GW4869 is an inhibitor of neutral sphingomyelinase 2, an enzyme implicated in ceramide biosynthesis.[51−53]

The secretion of γ-synuclein-GFP from SH-SY5Y cells (lanes 4−6) is significantly inhibited by both GW4869 [58 ± 5% inhibition; n = 3 (Figure 6E, lane 5)] and MBC [92 ± 5% inhibition; n = 3 (Figure 6E, lane 6)] compared to the control without inhibitors (lane 4). MBC inhibited secretion of the γ-synuclein monomer by 34 ± 5% (n = 3; lane 3), whereas no effect of GW4869 on the secretion of monomer γ-synuclein was found (Figure 6E, lane 2). The level of inhibition was more significant for γ-synuclein-GFP than for γ-synuclein without the tag. The amount of exosome marker CD63 was also reduced in the presence of both inhibitors (Figure 6 F). The average reduction in the level of CD63 by GW4869 was 46.2 ± 5% (n = 3; lane 5), and the average reduction caused by MBC was 73.6 ± 6% (n = 3; lane 6).

Intracellular Localization of the Secreted γ-Synuclein in Astrocytoma Cells. After U-87 astrocytoma cells had been grown in the presence of CM from SH-SY5Y stable clones overexpressing γ-synuclein, we observed the transmission of γ-synuclein into the cytoplasmic (Figure 7D,F) and nuclear (Figure 7A,C) compartments (arrows). PHA-L-lectin is localized in the Golgi compartment (Figure 7B, arrowhead). After 7 days, γ-synuclein is localized in the nucleus (Figure 7A,C, arrows) and cytoplasm (Figure 7D,F, arrows). If the cells are double stained before fixation, the lectin is localized exclusively in the cell membranes and no γ-synuclein staining is observed, pointing to intracellular localization of γ-synuclein.

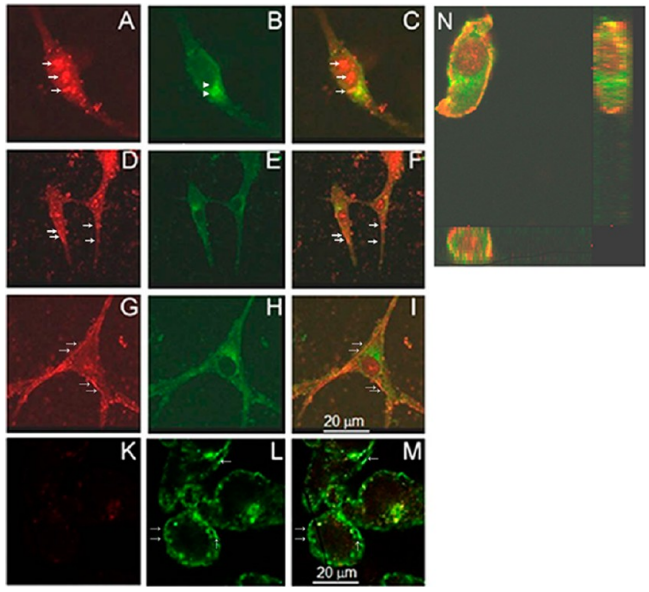

Figure 7. Fluorescence microscopy of astrocytoma U-87 cells: (A, D, G, and K) cells stained with γ-synuclein anti-rabbit polyclonal antibody (1:250) and anti-rabbit TRITC (1:150) as the secondary antibody (red), (B, E, H, and L) PHA-L conjugated with Alexa488 (green) (1:150), and (C, F, I, and M) double staining by both dyes. U-87 cells were grown for 7 days (A−F) and 12 days (G−I). (K−M) The cells were stained with PHA-L conjugated with Alexa488 and γ-synuclein anti-rabbit polyclonal antibody before fixation. Astrocytoma cells were grown in the presence of CM taken from SH-SY5Y neuronal cultures. γ-Synuclein is localized in the cytoplasm (D) and nucleus (A and C, arrow). PHL-lectin is confined to the Golgi compartment (B, arrowhead). After 12 days, the formation of fibrils localized mainly along the long axis of the cell can be observed (G, double arrow). Without the primary antibody, there is no staining (not shown). If the cells were double stained before fixation, the lectin was localized exclusively in the cell membranes and no γ-synuclein staining was observed, pointing to intracellular localization of γ-synuclein. The results of confocal microscopy with the analysis of z-stacks support the intracellular localization of γ-synuclein, part of which is confined to the nucleus (N).

After the culture had been stained for 12 days, the formation of fibrils localized mainly along the cell could be seen (Figure 7G,I, double arrows). Without the primary Ab, there is no staining (not shown). The results of confocal microscopy with the subsequent analysis of z-stacks also suggest nuclear localization of γ-synuclein (Figure 7N), confirming the data presented in panels A and C of Figure 7.

■ DISCUSSION

α-Synuclein has been linked to the pathogenesis of PD and other synucleinopathies through its propensity to form toxic aggregates, but the upstream events causing α-synuclein aggregation are not completely understood.

We demonstrate here for the first time that the propensity of α-synuclein to aggregate is enhanced by oxidized γ-synuclein (Figure 1). This property of γ-synuclein may play an important role in pathology, because these two proteins are colocalized in several parts of the brain, i.e., hippocampus, amygdale, brainstem, striatum, and frontal cortex.[54] Both α-synuclein and γ-synuclein are expressed in motoneurons, peripheral sensory neurons,[55] primary raphae nuclei neurons,[56] and other cell types. The existence of heteromeric protein−protein complexes between γ-synuclein and α-synuclein that we

demonstrate here by WB (Figure 1F) was revealed previously by co-immunoprecipitation.[56] The presence of heterodimers suggests that the interaction between several members of this family may play an essential role in the regulation of their aggregational status. Interestingly, unmodified γ-synuclein and β-synuclein possess chaperonic activity[42,57] and can inhibit α-synuclein fibrillation,[58−60] so oxidative stress and post-translational modifications significantly change synuclein's functional properties.

The body of evidence that pathological interactions among different proteins that are prone to aggregate play an essential role in the formation of neurotoxic oligomers that accumulate in neurons or glial cells is growing. Earlier the interaction between different naturally unfolded proteins has been described for amyloid β (Aβ) and α-synuclein,[61−63] tau and α-synuclein,[64] and Aβ and tau.[65] The broader implications of such hybrid interactions might be important in the pathogenesis of other protein misfolding diseases.

A growing body of published evidence reveals the considerable overlap of clinicopathological features among NDDs. This overlap can be explained by the formation of hybrid misfolded protein oligomers.[61−66] Oxidative stress is an important contributing factor in the pathogenesis of many NDDs. The level of oxidatively modified proteins in PD and synucleinopathies is increased, leading to the impairment of several cellular functions.[67,68]

We show here that γ-synuclein is the most easily oxidized member of the synuclein family and that it forms aggregates after oxidation. Aggregates can be detected by Coomassie staining (Figure 3A, lane 3) and WB (Figure 1B, lane 9). Importantly, oxidized γ-synuclein seeds α-synuclein aggregation both in vitro (Figure 1C,D) and in vivo (Figure 5C,D). Easy oxidation of Met[38] and Tyr[39] in γ-synuclein may be explained by the peculiarities of its amino acid sequence and secondary structure. In α-synuclein, the corresponding positions are occupied by Leu[38] and Tyr[39] (Figure 1A) located in a linker between two α-helices.[69] γ-Synuclein has a similar molecular structure except for the C-terminal part of the molecule (figure in the Table of Contents).[70] The linker is a loosely packed and surface-exposed region that is more readily accessible to oxygen.[71] Interestingly, the linker between two helices is located on the boundary between the first and second exons, and its amino acid sequence is conserved in human, cow, mouse, rat, porcine, and *Xenopus* α-synuclein,[70] suggesting that such a structural feature is preserved during evolution. Localization of Met[38] in a position proximal to Tyr[39] allows intramolecular oxygen transfer and enhances the propensity to aggregate.[48] Tyr[39] plays a critical role in α-synuclein fibrillation,[10,72] and its oxidative modification affects its structure and aggregational properties.[73]Methionine residues are important for the long-range interactions and secondary structure of α-synuclein. Furthermore, oxidation of methionine residues in α-synuclein affects electrostatic and hydrophobic interactions and the propensity to fibrillate.[74]

We have found that the substitution of Met[38] or Tyr[39] with Ala reduces the level of aggregation of the protein. Thus, the propensity of γ-synuclein to aggregate under oxidative conditions in vitro depends on Met[38] and Tyr[39] localized in adjacent positions. This structural property together with the presence of a hydrophobic stretch of amino acids (NAC domain) in the central part of its molecule (Figure 1A) presumably facilitates α-synuclein aggregation and its ability to induce the aggregation of α-synuclein.

In another member of the synuclein family, α-synuclein, Tyr[39] plays a critical role in its fibrillation,[46,48] while in proteins with Tyr and Met in neighboring positions, an efficient intramolecular oxygen transfer occurs to give a dioxygenated derivative with one oxygen on the Tyr and the other forming methionine sulfoxides. In this case, Tyr may play a catalytical role to produce Met oxidation.[48]

The annular forms of oxidized γ-synuclein that we have described here might have other deleterious effect on cell viability affecting membrane permeability as shown previously for α-synuclein and other amyloid proteins.[49,75−77] Some α-synuclein aggregates seeded by γ-synuclein also have annular structure (Figure 4E, panels i−k), suggesting that γ-synuclein oxidation may trigger the accumulation of porelike synuclein aggregates. Synuclein-containing porelike structures within cell membranes may cause a disruption of cellular ion homeostasis, including channel-mediated calcium influx and subsequent cell death.[63,75−77]

Oxidized γ-synuclein accumulates in neuronal cells and forms intracellular inclusions (Figure 5A,B). Furthermore, γ-synuclein can be secreted from cells into CM in aggregated form (Figure 6). In the presence of proteasome inhibitors, higher-molecular mass aggregates are formed (Figure 6A). Aggregated γ-synuclein can be internalized by astrocytoma cells (Figure 6D). This process is accompanied by the alteration of the pattern of aggregation, which may be explained by changes in protein conformation and/or post-translational modifications.

In the presence of a cholesterol-depleting drug, MBC, and an inhibitor of ceramide biosynthesis, GW4869, the extent of γ-synuclein secretion as well as the amount of exosome marker CD63 was decreased (Figure 6E), further confirming that γ-synuclein export takes place through exosomes.

The ability of γ-synuclein to seed α-synuclein aggregation found in vitro (Figure 1) was reproduced in experiments with cell cultures (Figure 5). However, when we analyzed the ability of γ-synuclein to seed α-synuclein aggregation in vitro and in cell cultures, we revealed some differences. For example, DA-treated γ-synuclein seeded α-synuclein aggregation more efficiently than the intact protein in vitro (Figure 1), whereas no differences were found in cell cultures (Figure 1E, lanes 2 and 3). These discrepancies may be explained by γ-synuclein post-translational modifications that change its properties in the cellular milieu.

The discovery of α-synuclein in extracellular biological fluids, including human CSF and blood plasma, in both healthy subjects and patients with PD[11−13] suggests that extracellular α-synuclein may physiologically or pathologically affect both neuronal and glial brain cells.[26]Importantly, the levels of oligomeric α-synuclein in CSF and blood plasma are elevated in PD patients.[12,13] Secretion of α-synuclein into extracellular fluids is an important first step in the neuron to neuron or neuron to glial cell propagation of pathology in NDDs.[16,28,80]

The data presented here show that γ-synuclein also participates in secretion and internalization. It can be secreted into CM and internalized by neuronal or glial cells, resulting in the intracellular accumulation and deposition of γ-synuclein. In addition, oxidized γ-synuclein seeds α-synuclein aggregation and therefore possesses prion-like properties. Thus, synucleins exert their pathogenic actions not only in the cytoplasm of neuronal cells that strongly express these proteins but also in the extracellular space affecting the function of neighboring cells. These results provide new insights into the events that regulate the formation of intracellular aggregated α-synuclein

and further elucidate the pathological mechanisms underlying PD and other synucleinopathies.

Recent data suggest that under physiologic conditions, α-synuclein exists in a tetrameric form that has considerable helical content.[78,79] Our results indicate that γ-synuclein is present in strongly overexpressing cells as a SDS-resistant tetramer. These new findings demonstrate that further studies are required to improve our understanding of factors regulating the state of synuclein aggregation. Among these factors are post-translational modifications of synucleins and their interaction with other proteins as well as with small molecules that could affect the level of aggregation and change synuclein pathogenicity.

A better understanding of the steps involved in the process of α-synuclein aggregation and the role of γ-synuclein as a core inducing such aggregation is important for developing intervention strategies that would prevent or reverse the accumulation of toxic proteins in NDDs.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

Ab, antibody; CE, cell extracts; CM, conditioned medium; DA, dopamine; DMEM, Dulbecco's modified Eagle's medium; MBC, methyl-β-cyclodextrin; NDD, neurodegenerative diseases; PAAG, polyacrylamide gel; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PD, Parkinson's disease; PFA, paraformaldehyde; RT, room temperature; TEM, transmission electron microscopy; WB, Western blotting; wt, wild type.

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