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Cystatin C Triggers Neuronal Degeneration in a Model of Multiple System Atrophy

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Multiple system atrophy is an intractable neurodegenerative disease caused by α -synuclein (α -syn) accumulation in oligodendrocytes and neurons. With the use of a transgenic mouse model overexpressing human α -syn in oligodendrocytes, we demonstrated that oligodendrocytic α -syn inclusions induce neuronal α -syn accumulation, resulting in progressive neuronal degeneration. The mechanism through which oligodendrocytic α -syn inclusions trigger neuronal α -syn accumulation leading to multiple system atrophy is unknown. In this study, we identified cystatin C, an oligodendrocyte-derived secretory protein that triggers α -syn up-regulation and insoluble α -syn accumulation, in neurons of the mouse central nervous system. Cystatin C was released by mouse oligodendrocytes overexpressing human α -syn, and extracellular cystatin C increased the expression of the endogenous α -syn gene in wild-type mouse neurons. These neurons then accumulate insoluble α -syn and may undergo apoptosis. Cystatin C is a potential pathogenic signal triggering neurodegeneration in multiple system atrophy. (*Am J Pathol* 2014, 184: 790–799; <http://dx.doi.org/10.1016/j.ajpath.2013.11.018>)

Oligodendrocytes surround and ensheath neuronal axons to support action potential propagation. Oligodendrocytes and neurons also communicate by releasing chemical signals that allow functional coupling of axonal activity and oligodendroglial function. Moreover, signals released from dysfunctional oligodendrocytes may contribute to neuronal degeneration.¹ Multiple system atrophy (MSA) is a neurodegenerative disease caused by α -synuclein (α -syn) accumulation in oligodendrocytes and neurons and is clinically characterized by various combinations of parkinsonism, cerebellar dysfunction, and autonomic nervous system failure.^{2–4} No effective therapy is available for MSA-associated neurodegeneration.

Two pathological hallmarks of MSA are glial cytoplasmic inclusions (GCIs) and neuronal inclusions, both of which are composed of α -syn. The GCIs are confined to oligodendrocytes and are the first neuropathological manifestations of MSA, preceding the neuronal inclusions that ultimately lead to neurodegeneration and neurological deficits.^{5–10} Mouse models of MSA have revealed that α -syn accumulation in oligodendrocytes contributes to progressive neuronal degeneration in the central nervous system (CNS).^{11–13} We previously demonstrated that transgenic (Tg) mice expressing

human α -syn in oligodendrocytes under the control of the 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) promoter exhibited motor impairments, progressive brain atrophy, and GCI-like α -syn inclusions similar to those described in patients with MSA.¹³ These Tg mice not only recapitulated many features of MSA neuropathology but also provided evidence that formation of oligodendrocytic α -syn inclusions results in neuronal accumulation of insoluble α -syn, leading to neuronal dysfunction and degeneration in the mouse CNS.^{13,14} This insoluble α -syn accumulates in the CNS by binding to the neuron-specific microtubule protein β -III tubulin.^{15,16} Although the binding of neuronal α -syn to β -III tubulin is a key process that leads to neuronal degeneration in the MSA mouse model, it is still unclear how oligodendrocytic α -syn inclusions trigger neuronal degeneration.

In a subsequent study, we found that the conditioned media (CM) from Tg mouse brain cultures induced insoluble α -syn accumulation in cultured neurons from non-Tg mice.¹⁵ It has been proposed that an oligodendrocyte-derived signal may trigger the accumulation of insoluble α -syn in neurons,

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resulting in the progressive neurodegeneration characteristic of MSA. Here, we identified cystatin C as an oligodendrocytic signal that causes neuronal degeneration. Oligodendrocytes overexpressing α -syn released cystatin C. Treatment of non-Tg mouse neural cultures with cystatin C up-regulated α -syn expression and led to the accumulation of insoluble α -syn in neurons, strongly suggesting that cystatin C is an oligodendrocytic signal that mediates neurodegeneration in MSA.

Materials and Methods

Tg Mice and Primary Culture

Tg mice were generated to express human wild-type α -syn in oligodendrocytes under the control of the murine CNP promoter.¹³ Primary cultures of neurons and glial cells were prepared as previously described¹⁵ with minor modifications. Briefly, cerebral cortices were dissected from P0 to P1 non-Tg and Tg mice of either sex and dissociated. The cells were plated on 15-mm glass coverslips coated with polyethylenimine (0.01%; Sigma-Aldrich, St. Louis, MO) at a density of 0.6×10^5 cells/cm² for immunocytochemistry or on polyethylenimine-coated 75-cm² flasks at 2.0×10^5 cells/cm² for preparation of CM and biochemical experiments. Cells were maintained in a mixture of Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) plus B27/neurobasal medium (1:1) supplemented with 5% fetal bovine serum, penicillin (50 U/mL), streptomycin (50 μ g/mL), glutamine (1 mmol/L), and insulin (50 μ g/mL; Sigma-Aldrich). Neuronal and glial composition of the neural cultures at 36 days *in vitro* (DIV) was confirmed by immunocytochemistry; in non-Tg mouse cell cultures, 10.4% $\pm$ 4.2% of all cells were Tuj1-positive neurons, 23.5% $\pm$ 7.6% were O4-positive oligodendrocytes, 42.0% $\pm$ 6.5% were glial fibrillary acidic protein-positive astrocytes, and 4.3% $\pm$ 2.0% were Iba1-positive microglia; in Tg mouse cell cultures, 10.3% $\pm$ 6.3% of all cells were Tuj1 positive, 23.4% $\pm$ 5.3% were O4 positive, 44.0% $\pm$ 8.1% were glial fibrillary acidic protein positive, and 4.7% $\pm$ 0.9% were Iba1 positive. To assay the effect of cystatin C on neurons, primary cultured cells were treated with purified cystatin C (0.1 to 2 μ g/mL) for 28 days from DIV8. To obtain a nearly pure culture of neurons, after 2 days in neural cultures, the cells were exposed to 1- β -D-arabinofuranosylcytosine (AraC; 10 μ mol/L; Sigma-Aldrich) for 3 days. AraC treatment resulted in a dramatic decrease in the number of glial cells in the cultures. All chemicals for culture were purchased from Life Technologies (Carlsbad, CA) unless otherwise stated. All experiments with mice were approved by the National Center for Geriatrics and Gerontology Animal Care and Use Committee.

Oligodendrocyte Culture

Oligodendrocyte cultures were obtained by a modified procedure of McCarthy and de Vellis.¹⁷ Briefly, mixed glial cells were grown for 9 to 11 days. To remove microglial

cells, primary cultures were shaken rotationally for 1 hour at 160 rpm at 37°C. The medium was discarded, and fresh medium was added. Cultures were shaken for 20 hours at 220 rpm at 37°C in which oligodendrocytes were dislodged from the underlying astrocyte layer. Cells were harvested by centrifugation, resuspended in growth medium, and grown for 14 days on poly-L-lysine-coated glass coverslips. Oligodendrocyte cultures did not contain neuronal cells, and astrocytic contamination was <10%.

Isolation of Signaling Molecules

Half of the media from each primary culture derived from Tg and non-Tg mouse brain was collected twice weekly from DIV7 to DIV40 and replaced with new medium. Samples of CM were fractionated and concentrated by sequential ultrafiltration on Vivaspin20 columns (Sartorius, Goettingen, Germany). First, CM (1 L) were filtered through a 100,000-molecular weight cutoff (MWCO) membrane to remove cell debris and high molecular weight proteins. The fraction passed through the 100,000-MWCO membrane was concentrated with a 50,000-MWCO membrane. The concentrated fraction in 1.5 mL of CM was supplemented with 20 mmol/L Tris-HCl (pH 7.5) to 10 mL, and the mixture was loaded at 1 mL/minute onto a MonoQ 46/100 PE column (GE Healthcare, Buckinghamshire, UK) pre-equilibrated with 20 mmol/L Tris-HCl (pH 7.5). The flow-through fraction was collected and concentrated by ultrafiltration through a 5000-MWCO membrane. The flow-through fraction was then loaded onto a HiLoad 16/60 Superdex 200 column (GE Healthcare) pre-equilibrated with 20 mmol/L Tris-HCl (pH 7.5) containing 0.15 mol/L NaCl and eluted with the same buffer at 0.8 mL/minute. The eluting proteins were monitored by absorbance at 280 nm and collected in 3-mL fractions. The protein content of fractions was assessed by SDS-PAGE and silver staining.

In-Gel Digestion and Mass Spectrometry

Each Superdex 200 fraction from CM of Tg and non-Tg cells was separated on 12% SDS-polyacrylamide gels and visualized with the Silver Stain MS Kit (Wako). Protein bands specific to the CM from Tg mice were excised from silver-stained gels and subjected to in-gel trypsin digestion with the use of the Trypsin Profile IGD Kit (Sigma-Aldrich) according to the manufacturer's instructions. The mixture of tryptic peptides was concentrated, desalted on a ZipTipC18 microcolumn (Millipore, Billerica, MA), and subsequently analyzed by an AXIMA-CFR matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometer (Shimadzu-Kratos, Kyoto, Japan) by using α -cyano-4-hydroxycinnamic acid as the matrix. The peptide-mass fingerprinting results generated from MALDI-TOF mass spectroscopy (MS) were used to identify the protein by searching the SWISS-PROT database by using the MASCOT peptide-mass fingerprinting search program (Matrix Science, Inc., Boston,

http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF, last accessed July 29, 2011).

Real-Time PCR Analysis

Harvested tissues and cells were immediately soaked in RNeasy lysis reagent (Qiagen, Hilden, Germany) and frozen. Total RNA was isolated from tissues and cells by using NucleoSpin RNA II (Takara Bio, Shiga, Japan). Purified total RNA (1 µg) was converted to cDNA with the use of the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Gene expression levels were quantified with Power SYBR Green PCR Master Mix (Life Technologies). The primer sequences are as follows: *α*-syn (*SNCA*) forward, 5'-TCGACGACAGTGTGGTGTAAAGG-3', *SNCA* reverse, 5'-CACCATGCACCACTCCCTCC-3'; *Snca* forward, 5'-GCCTGTGGATCCTGGCAGT-3', *Snca* reverse, 5'-CGCCATGGAAGAGCAG-3'; cystatin C (*Cst3*) forward, 5'-GCGTACCAC-3', *Cst3* reverse, 5'-TGGGGCTGGTC-3'; Bcl2-associated protein X (*Bax*) forward, 5'-CCGAGCTGATCAGAACCATCA-3'; *Bax* reverse, 5'-CTCCAGCCACAAAGATGGTCA-3'; caspase-3 (*Casp3*) forward, 5'-ACAGTGGGACTGATGAG-3', *Casp3* reverse, 5'-TGCAAAGGGACTGGAT-3'; ubiquitin C (*Ubc*) forward, 5'-CACAGCCCT-3', *Ubc* reverse, 5'-TGGTGTCACT-3'; *Gapdh* forward, 5'-AGCAAG-3', *Gapdh* reverse, 5'-TGGT-3'; mitochondrial ribo-

somal protein L4 (*Mrpl4*) forward, 5'-AGACCAGGGCT-3', *Mrpl4* reverse, 5'-GCCATAAGG-3'. Relative RNA levels were normalized to ubiquitin C expression.¹⁸ Stable housekeeping genes *Gapdh* and *Mrpl4* were used as internal controls.

Knockdown of *Cst3* and *Snca* by RNA Interference

For siRNA knockdown of *Cst3* or *Snca*, lentiviral vector-based siRNA plasmids (piLenti-siRNA-GFP) that contained target sequences were purchased from Applied Biological Materials Inc. (Vancouver, Canada). The target sequences of the siRNAs are as follows: *Cst3*-1, 5'-GCAGCTCGTG-3', *Cst3*-2, 5'-TGACTGA-3', *Snca*-1, 5'-ACAG-3', *Snca*-2, 5'-AGGAAGGCTACCAAGACTATGAGCCTGAA-3'. These plasmids were transfected into primary cultured cells with the use of Lipofectamine 2000 (Life Technologies). After 14 days of transfection, cells were harvested for analysis.

Generation of Stable Cell Line and Purification of Secreted Cystatin C

The complete cDNA sequences of the *Cst3* gene was obtained by RT-PCR cloning with the use of total RNA isolated from the brain tissue of a 6-month-old non-Tg mouse as a template. To fuse a FLAG tag sequence to the 3' region of the *Cst3* gene, PCR was performed with primers that contained the tag sequence. The PCR products were subcloned into pT7Blue

Figure 1 Isolation and identification of candidate signaling molecules to regulate neuronal *α*-syn accumulation. **A:** CM samples from primary neural cultures were fractionated by sequential ultrafiltration and monoQ chromatography. The collected proteins were fractionated by Superdex 200 gel filtration chromatography. Protein concentrations in the CM from non-Tg (diamonds) and Tg (circles) mice were estimated by absorbance at 280 nm. **B:** Fractions 13 to 22 were separated by SDS-PAGE, and the proteins were visualized by silver staining; CM were derived from non-Tg (left panel) and Tg mice (right panel). A 14.9-kDa band (arrow) was specifically detected in fraction 18 obtained from Tg mouse primary cells and was excised from the SDS-polyacrylamide gel for mass spectrometric analysis. **C:** The 14.9-kDa band was subjected to in-gel digestion with trypsin, and the extracted peptides were analyzed by MALDI-TOF MS. **D:** Five peptide masses (1256.63, 1412.62, 1542.53, 1811.65, and 1939.65) matched mouse cystatin C. **E:** The tryptic peptides (bold) cover 42 of 140 amino acids of the cystatin C protein.

(Merck, Darmstadt, Germany). To generate stable cell lines, Cst3 cDNA was first cloned into the pDsRed-Monomer-Hygro-C1 vector (Takara Bio). COS-7 cells maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum were transfected with the Cst3

vector by using FuGENE 6 (Roche Applied Science, Basel, Switzerland) according to the manufacturer's instructions. To select stable clones, transfected cells were cultured for 2 weeks in 400 µg/mL hygromycin B (Life Technologies), and surviving colonies were transferred to separate culture dishes and

Figure 2 Up-regulation of *Cst3* expression in the Tg-mouse CNS. **A** and **B**: The mRNAs of *Cst3*, *Snca*, and *SNCA* in cultured neuronal and glial cells at DIV36 (**A**) and brain tissues of Tg and non-Tg mice at 12 months of age (**B**) were measured by qPCR. The expression of *Cst3* and *Snca* mRNAs in Tg mice is shown as a ratio to that in non-Tg mice. **C**: Age-dependent changes in the relative expression levels of *Snca* and *Cst3* mRNAs were analyzed by qPCR. *Gapdh* mRNA was used as an internal control. Data are expressed as the means $\pm$ SD ($n = 3$). * $P < 0.05$, Student's *t*-test. **D**: Cultured neuronal and glial cells from Tg and non-Tg mice were fixed at DIV36 and immunostained for cystatin C (green) and oligodendrocyte marker CNP (red). Cell nuclei were counterstained with DAPI (blue). **E**: The cells were immunostained for cystatin C (green) and neuronal marker Tuj1 (red). Double labeling showed that cystatin C was localized in the oligodendrocytic cytoplasm. Cystatin C immunoreactivity was stronger in oligodendrocytes derived from Tg mice than oligodendrocytes from non-Tg mice. **F**: Oligodendrocyte cultures from Tg and non-Tg mice were fixed at DIV25 and immunostained for cystatin C (green) and the oligodendrocyte marker (red). Cystatin C was localized in the oligodendrocytic cytoplasm. The ratio of cystatin C-positive cells in the oligodendrocytes was calculated. The mean percentage of cystatin C-positive cells from five different microscopic fields is plotted. Data are expressed as the means $\pm$ SD. * $P < 0.05$, Student's *t*-tests. **G**: Sections of the spinal cord from Tg and non-Tg mice at the age of 12 months were stained by immunofluorescence with cystatin C (green) and CNP (red). **H**: The sections were also immunostained with cystatin C and developed with diaminobenzidine. Cystatin C was detected in oligodendrocytes of the Tg mouse CNS (arrows). Scale bars: 10 µm (**D–H**).

grown in 200 µg/mL hygromycin B medium. Expression and secretion of cystatin C were confirmed in each line by immunoblot analysis. To obtain secreted cystatin C, the cultured medium from these COS-7 cells was collected and dialyzed against 20 mmol/L Tris-HCl (pH 7.5). The protein was then further concentrated by lyophilization. After partial purification with the use of ultrafiltration, cystatin C was purified by Superdex 200 gel filtration chromatography.

Transient Expression of α -Syn in Neuro2a Cells

The mouse α -syn gene *Snca* was cloned into the pcDNA3.1 vector as previously described,¹⁵ and FuGENE 6 was used to introduce exogenous DNA into Neuro2a cells.¹⁹ To induce neuronal differentiation of Neuro2a cells, the medium was replaced with Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum and 20 µmol/L retinoic acid (Sigma-Aldrich). To assay the effect of cystatin C on differentiated Neuro2a, cells were treated with cystatin C (1 µg/mL) for 7 days.

Sequential Extraction of α -Syn from Cultured Cells

Cultured cells were homogenized in high-salt buffer (50 mmol/L Tris-HCl, pH 7.5, 750 mmol/L NaCl, 20 mmol/L NaF, and 10 mmol/L EGTA) at 0.5 mL per culture flask and centrifuged at $100,000 \times g$ for 30 minutes at 4°C. The supernatant was the high-salt-soluble fraction. The resulting pellet was suspended in 50 mmol/L Tris-HCl that contained 10% (w/v) sucrose (pH 7.5) to remove myelin and associated lipids. The remaining pellet was dissolved in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 5 mmol/L EGTA, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS] and centrifuged at $100,000 \times g$ for 30 minutes at 4°C. The radioimmunoprecipitation assay buffer-insoluble proteins were extracted from the pellet by sonication in 70% (v/v) formic acid (FA). Samples extracted by FA were dried and resuspended in dissociation buffer [0.5 mol/L Tris-HCl, pH 8.8, 1% (w/v) SDS, 5% (v/v) 2-mercaptoethanol]. Protein concentrations were measured by the bicinchoninic acid protein assay (Thermo Fisher Scientific, Waltham, MA). Equal amounts of samples were separated by SDS-PAGE and analyzed by immunoblot analysis.

Immunocytochemical and Immunohistochemical Studies

Primary cultured cells and Neuro2a cells on glass coverslips were fixed and stained as previously described.¹⁵ The primary antibodies used were Syn4469, anti-cystatin C (Millipore), anti-cleaved caspase-3 (Asp175; Cell Signaling Technology, Beverly, MA), anti-CNP (Lab Vision, Fremont, CA), anti-O4 (Millipore), anti- β -III tubulin (Tuj1; Covance, Princeton, NJ), anti-glial fibrillary acidic protein (Sigma-Aldrich), and anti-Iba1 (Wako). Syn4469 is a polyclonal antibody that

selectively recognizes mouse α -syn residues 115 to 125.¹⁵ After incubation with a primary antibody, the cells were incubated for 1 hour with a fluorophore-conjugated secondary antibody (Alexa 488- or Alexa 594-conjugated goat anti-mouse IgG or anti-rabbit IgG; Life Technologies). *In situ* labeling of fragmented genomic DNA in primary cultured cells was performed with the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Applied Science) according to the manufacturer's instructions. TUNEL-positive cells were counted by a blinded observer and the average was calculated.

For immunohistochemistry, anesthetized mice were perfused transcardially with PBS. Mouse brains were fixed in 10% neutral-buffered formalin and were sectioned. Sections were blocked with 2% fetal bovine serum in 0.1 mol/L Tris-HCl (pH 7.5) and incubated overnight at 4°C with primary antibodies. Sections were reacted with biotinylated-conjugated secondary antibodies and developed with diaminobenzidine by using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA), as previously described.²⁰ A double-labeling immunofluorescence study was conducted to evaluate the colocalization of oligodendrocytes and cystatin C in the mouse CNS. Nuclei were visualized by counterstaining with DAPI.

Immunoblot Analysis

Samples were electrophoretically separated on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (GE Healthcare). The polyvinylidene difluoride membranes were incubated in 5% nonfat milk powder in Tris-buffered saline and then incubated with Syn4469 and anti-160-kDa neurofilament (NF160; Sigma-Aldrich), followed by incubation in a horseradish peroxidase-conjugated secondary antibody. To enhance the immunoreactions, we used the Can Get Signal Immunostain Solution (Toyobo, Osaka, Japan) to dilute primary and secondary antibodies. Immunoreactive proteins were visualized with an electrochemiluminescence plus system (GE Healthcare). Densitometric measurement of the signals was performed with ImageJ software version 1.43u (NIH, Bethesda, MD).

Statistical Analysis

Data are expressed as means $\pm$ SD. Statistical analysis was performed by Student's *t*-test. A *P* value of <0.05 was considered to be statistically significant.

Results

Identification of an Oligodendrocytic Signaling Molecule that Triggers Neuronal α -Syn Accumulation in a Mouse Model of MSA

To identify oligodendrocytic signaling molecules that can trigger neuronal degeneration in the MSA mouse model, CM samples from primary cultures of Tg and non-Tg

mouse brains were fractionated, and the molecular constituents were compared. After separation of CM by ultrafiltration and anion exchange chromatography, the flow-through solution was fractionated with the use of size exclusion chromatography (Figure 1A). We observed a distinct peak in the elution profile that distinguished the CM of Tg mice from that of non-Tg mice. The protein fractions within this peak were separated by SDS-PAGE

and visualized by silver staining (Figure 1B). We observed a distinct band at 14.9 kDa in fraction 18 of Tg mice CM. This band was excised from the gel and digested with trypsin. The protein fragments were analyzed by MALDI-TOF MS (Figure 1C) and identified by searching the SWISS-PROT database with the MASCOT program (Figure 1, D and E). In this way, we identified the protein present in the 14.9-kDa band as cystatin C.

Figure 3 Accumulation of insoluble α -syn in neurons treated with cystatin C. **A:** Primary cultured neural cells derived from non-Tg mice were treated with cystatin C from DIV8 to DIV36 and compared with untreated cultures. Expression of α -syn was quantified by qPCR and immunoblot analysis. A dose—response curve of Snca mRNA expression in response to cystatin C was plotted. Mrpl4 mRNA was used as an internal control. α -Syn signals on the immunoblots were normalized to the signals for β I-tubulin. **B:** Expression of cystatin C was suppressed by RNA interference in primary neural cells derived from Tg mice. Two siRNA vectors (Cst3-1 and Cst3-2) or control vector were introduced into the primary neural cells. Expressions of Cst3 and Snca mRNAs were determined by qPCR, and protein depletions were assessed by immunoblots and their quantification. CNP and β III-tubulin were used as protein markers for oligodendrocytes and neurons, respectively, in immunoblots. β I-tubulin was used as a loading control. Data are expressed as the means $\pm$ SD ($n = 3$). $*P < 0.05$ by Student's t -tests. **C:** Proteins from primary cultured neural cells derived from non-Tg mice (control and treated with cystatin C) and Tg mice were sequentially extracted into three fractions: high-salt (HS) buffer, radioimmunoprecipitation assay (RIPA) buffer, and 70% formic acid (FA). Neurofilament (NF160) was used as a protein marker for the neuronal insoluble protein fraction.

Elevated Cystatin C Expression by Oligodendrocytes of the MSA Mouse Model

To determine whether α -syn overexpression in oligodendrocytes contributes to cystatin C release in the Tg mouse CNS, we examined the age-dependent expression of cystatin C and α -syn by quantitative real-time PCR (qPCR). Expression levels of the mouse *Cst3* and the endogenous *Snca* genes were significantly higher in Tg mouse neural cells than in non-Tg mouse neural cells (Figure 2A). Similarly, the expression of *Cst3* and *Snca* mRNAs was up-regulated in the brain tissue of Tg mice at 12 months of age (Figure 2B). We then examined the expression of *Cst3* and *Snca* mRNAs in the brains of Tg and non-Tg mice at 3, 6, 12, and 18 months of age by qPCR. Overexpression of the human *SNCA* gene in oligodendrocytes was detected in Tg mouse brain at all ages from 3 months to 18 months. Inclusions that resembled GCIs were first detected at 6 months of age in the Tg CNS,¹³ subsequent to *Cst3* mRNA expression but preceding the up-regulation of *Snca* mRNA expression at 12 months of age (Figure 2C).

Cystatin C expression has been reported in neurons, astrocytes, and microglia of several species,^{21–25} but it is unclear whether cystatin C is expressed by oligodendrocytes. Neural cells cultured from Tg mice exhibited stronger immunocytochemical staining for cystatin C than neural cells cultured from non-Tg mice, and it was selectively expressed by oligodendrocytes (Figure 2, D and E). Oligodendrocyte cultures from Tg mice confirmed that cystatin C was localized at the cytoplasm (Figure 2F). Immunohistochemical studies revealed that cystatin C was localized to the oligodendrocyte cytoplasm in the Tg mouse CNS (Figure 2, G and H). These results indicate that cystatin C is produced by oligodendrocytes that contained α -syn inclusions.

Induction of Neuronal α -Syn Accumulation by Cystatin C

We previously reported that insoluble endogenous α -syn progressively accumulated in neurons, leading to neuronal degeneration in Tg mice.^{13,15} To examine the up-regulation of *Snca* mRNA by cystatin C, primary cultured cells derived from non-Tg mice were exposed to a dose (0.1 to 2 μ g/mL) of cystatin C for DIV8 to DIV36 (Figure 3A). We found that a 1- μ g/mL concentration of cystatin C was sufficient to stimulate a significant increase in *Snca* mRNA expression. To assess the effect of cystatin C on protein synthesis of α -syn, primary neural cultures from non-Tg mice were treated with 1 μ g/mL cystatin C and compared with untreated neural cultures by immunoblot analysis. Both qPCR and immunoblot analysis revealed neuronal accumulation of endogenous α -syn induced by cystatin C treatment (Figure 3A). Moreover, knockdown of *Cst3* in Tg mouse cells resulted in the down-regulation of *Snca* mRNA expression (Figure 3B). We further analyzed the biochemical properties of α -syn in cultured neural cells treated with cystatin C. Sequential protein extraction showed that most α -syn

Figure 4 Effect of cystatin C on neuronal α -syn accumulation. **A–K:** Primary cultured neural cells derived from non-Tg mice were treated with cystatin C (**D–F**) and compared with untreated cells (**A–C**) and untreated Tg mouse cells (**G–I**). The cells were exposed to AraC to enrich neurons in the culture. Enriched neuronal cultures were treated with (**K**) or without (**J**) cystatin C. Primary neural cultures and enriched neuronal cultures were fixed and studied by immunofluorescence. Double-labeling immunocytochemistry revealed that the up-regulation of α -syn expression in response to cystatin C treatment was restricted to neurons, particularly axons. **L–T:** Neuro2a cells overexpressing mouse α -syn were followed by differentiation (**L–N**), and Neuro2a cells treated with cystatin C for 7 days (**R–T**) and untreated (**O–Q**) after differentiation. When α -syn was overexpressed by transfection, α -syn protein was diffusely distributed throughout the cytoplasm and neurites, consistent with a soluble protein. In contrast, α -syn expressed after cystatin C treatment formed clusters in the neurites. Cells were stained with anti-mouse α -syn antibody (green) and an antibody against the neuron-specific β -III tubulin, Tuj1 (red). The area highlighted by the white box is enlarged in the right panels. Nuclei were counterstained with DAPI (blue). Scale bars: 10 μ m (**H, I, K, M, N, S, and T**).

Figure 5 Apoptotic neuronal degeneration induced by cystatin C. Primary neural cells derived from non-Tg mice were treated with cystatin C from DIV8 to DIV36. **A:** Elevated expression levels of Bax and Casp3 mRNAs in the cultured cells were quantified by qPCR. Cystatin C treatment increased the immunoreactivity of the cleaved caspase-3 on immunoblots stained by the antibody against cleaved caspase-3 (c-casp3). Double-labeling immunofluorescence showed that neurons (red) were selectively stained by the c-casp3 antibody (green). **B:** Two siRNA vectors (Snca-1 and Snca-2) or control vector with GFP were introduced into the primary neural cells, and expression of Snca mRNA was determined by qPCR and quantified. The c-casp3 was examined by immunofluorescence to assess neurotoxicity in the primary neurons by using the antibody against c-casp3 (red) and Tuj1 (blue). **C:** TUNEL staining to detect apoptotic cells (green) was performed on non-Tg primary cells treated with cystatin C. Neurons were identified by double-labeling with Tuj1. The cells undergoing apoptotic cell death were primarily neurons. **D:** The ratio of TUNEL-positive neurons in all neurons was calculated. The mean percentage of TUNEL-positive nuclei from 10 different microscopic fields is plotted. Data are expressed as the means $\pm$ SD. * P < 0.05, ** P < 0.01, Student's t -tests. Scale bars: 10 μ m (**A–C**). GFP, green fluorescent protein.

accumulation in Tg neurons was in the insoluble FA fraction, whereas cystatin C treatment enhanced insoluble α -syn accumulation in the FA fraction extracted from non-Tg neurons (Figure 3C).

To determine the intracellular localization of insoluble α -syn accumulation, primary neural cultures (Figure 4, A–I) and AraC-treated neuronal cultures (Figure 4, J and K) from non-Tg mice treated with cystatin C as described were immunocytochemically stained with an anti-synuclein antibody. Staining revealed accumulation of α -syn puncta in the soma and axons of these cystatin C-treated non-Tg neurons. This subcellular distribution was in marked contrast to the diffuse staining observed in the differentiated neuroblastoma line (Neuro2a) after transfection with mouse α -syn, a

staining pattern consistent with soluble α -syn expression (Figure 4, L–N). However, when untransfected Neuro2a cells were treated with cystatin C, α -syn staining appeared in clusters (Figure 4, O–T). The distinct staining patterns of Snca-transfected Neuro2a cells and cystatin C-treated Neuro2a cells indicated that cystatin C not only triggers the up-regulation of α -syn expression in neurons but also contributes to the accumulation of insoluble α -syn.

Neuronal Degeneration Induced by Cystatin C

To investigate whether cystatin C can induce neurodegeneration by enhancing insoluble α -syn accumulation, we compared the expression of apoptotic markers and

genomic DNA fragmentation between cystatin C-treated non-Tg neural cultures and control non-Tg neural cultures. qPCR revealed up-regulation of the *Bax* gene, a member of the Bcl-2 family that accelerates apoptosis, and the apoptosis effector gene *Casp3* in response to cystatin C treatment (Figure 5A). Immunofluorescence studies and immunoblot analysis showed that cystatin C activated caspase-3 in primary neurons (Figure 5A). When α -syn was down-regulated by RNA interference in the cystatin C-treated neurons, the cleaved caspase-3 was decreased (Figure 5B). TUNEL staining showed that cystatin C increased apoptosis (Figure 5, C and D). These results indicate that cystatin C is an oligodendrocytic signaling molecule that can induce neuronal degeneration in the mouse CNS.

Discussion

The interaction between oligodendrocytes and neurons plays a key role in the MSA neurodegeneration. Our study indicates that oligodendrocytes overexpressing human α -syn release signals that up-regulate neuronal expression of α -syn in an animal model of MSA. We identified cystatin C as one of these signals and demonstrated that this oligodendroglial protein triggered the accumulation of insoluble α -syn in neurons. We propose that cystatin C released by oligodendrocytes containing α -syn inclusions triggers neuronal degeneration in MSA.

Many neurodegenerative diseases are associated with the accumulation of specific proteins in the CNS, but the events that initiate this accumulation remain unclear. Intracellular transmission of exogenous protein aggregates is well documented in prion disorders.²⁶ In other neurodegenerative diseases associated with misfolded proteins such as β -amyloid (A β), tau, and α -syn, it has also been proposed that aggregates of misfolded proteins propagate the misfolded state in a prion-like manner.^{27–33} Growing evidence suggests that cell–cell interactions contribute to the spread of neurodegeneration, but the nature of the signals is still unclear. In this study, we show that the interaction between oligodendrocytes and neurons plays an essential role in the progressive neurodegeneration associated with MSA. In the mouse model of MSA, we clearly distinguished oligodendrocytic (exogenous) α -syn from neuronal (endogenous) α -syn. The exogenous α -syn in oligodendrocytes was of human origin, and the endogenous α -syn in neurons was of mouse origin in the mouse CNS.^{13,15} Thus, we speculate that oligodendrocyte-derived signals may serve as a bridge between oligodendrocytic inclusions and neurodegeneration in MSA. In another MSA mouse model, expression of glial-derived neurotrophic factor was reduced, whereas infusion of glial-derived neurotrophic factor ameliorated the behavioral and neuropathological deficits but had no effect on α -syn expression levels.³⁴ Because the accumulation of insoluble α -syn compromises neuronal function and viability, oligodendrocytes may release a multitude of signals with either trophic or deleterious effects on neurons. Our previous studies of the mouse model of MSA indicated that an oligodendrocyte-derived

signal must up-regulate α -syn in neurons, resulting in the accumulation of insoluble α -syn.¹⁵ Here, we identified cystatin C as one oligodendrocyte-derived signaling molecule that causes neuronal accumulation of α -syn in mouse CNS. We further need to clarify how cystatin C causes the neurotoxicity.

Cystatin C is a potent endogenous inhibitor of cysteine protease. It is also a secretory protein found in mammalian body fluids, including cerebrospinal fluid and tissues,³⁵ and is highly abundant in brain tissue.³⁶ A variant of cystatin C is the major constituent of amyloid deposited in the brains of patients with hereditary cerebral hemorrhage with amyloidosis, Icelandic type,³⁷ also called hereditary cystatin C amyloid angiopathy.³⁸ Overexpression of human cystatin C in the brains of Tg mice overexpressing the A β precursor protein reduces cerebral A β deposition, because cystatin C binds A β and inhibits its fibril formation.^{39,40} Conversely, cystatin C also regulates soluble A β and A β -associated neuronal defects by inhibiting cathepsin B-induced A β degradation *in vivo*.⁴¹ Thus, functional roles of cystatin C for amyloidosis are controversial.

The present study shows that cystatin C mediates the interaction between oligodendrocytes and neurons and is crucial for pathogenesis in the MSA mouse model. *Cst3* expression preceded the up-regulation of endogenous *Snca* expression, and the increase in endogenous *Snca* expression and intraneuronal α -syn accumulation corresponded to the onset of motor impairments in Tg mice.¹³ Moreover, the consistent delay between the oligodendrocytic *Cst3* up-regulation and neuronal *Snca* up-regulation suggests that accumulation of endogenous α -syn in neurons depends on oligodendrocytic *Cst3* up-regulation induced by GCI formation.

In conclusion, we demonstrated that oligodendrocytic cystatin C release induced by the formation of GCIs causes neuronal α -syn up-regulation and accumulation and may contribute to neuronal degeneration in a mouse model of MSA. Because cystatin C is an extracellular protein, it may have a unique potential for a novel therapeutic target for MSA.

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