이학석사학위논문

알파시뉴클레인 응집체의 세포 내 유입을 조절하는 새로운 막단백질의 분리

Isolation of novel membrane proteins regulating cellular uptake of α-synuclein aggregates

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ABSTRACT

Isolation of novel membrane proteins regulating cellular uptake of α-synuclein aggregates

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Recently, propagation of α-synuclein is of interest within the field of synucleinopathies, including Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy. However, little is known about membrane proteins which influence cellular uptake in the neuronal propagation of α-synuclein. Thus, to identify those membrane proteins, I have established a cell-based assay and screened membrane proteins. α-Synuclein was purified from *E. coli* and incubated *in vitro* for 21 days with sonication. Fibril forms of α-synuclein aggregates were confirmed by transmission electron microscope and labeled with Alexa Fluor 488 fluorescent dye. Cellular uptake of Alexa Flour 488-labeled α-synuclein

aggregates in SH-SY5Y cells was diminished by the treatment with hypertonic sucrose for inhibiting clathrin-mediated endocytosis, and 5-(N-Ethyl-N-isopropyl) amiloride for inhibiting macropinocytosis, supporting that some membrane proteins may regulate cellular uptake of α-synuclein aggregates. The cDNA expression library that encodes thousands of membrane proteins was prepared. SH-SY5Y cells grown on multi-well tissue culture plate were transfected with each of those cDNA and then exposed to α-synuclein aggregates. Nine putative cDNA clones were isolated to stimulate cellular uptake of α-synuclein aggregates. Among them, ectopic expression of STM1D9 or MTM7A8 was effective to stimulate cellular uptake of α-synuclein aggregates. On the other hand, overexpression of STM1D9 and MTM7A8 did not affect cellular uptake of tau aggregates and amyloid β oligomers. Moreover, ectopic expression of STM1D9 or MTM7A8 significantly increased cell-to-cell transmission of Venus1-α-synuclein and α-synuclein-Venus2 inSH-SY5Y/BiFC system. Conversely, knockdown of STM1D9 and MTM7A8 expression by sgRNA reduced cellular uptake of α-synuclein aggregates. These results suggest that STM1D9 and MTM7A8 might play a role in cellular uptake of α-synuclein aggregates during neuronal transmission.

Keywords : synucleinopathy, Parkinson's disease, α-synuclein, cell-to-cell transmission, propagation, internalization, receptor

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ABBREVIATIONS

AF488	**Alexa Fluor® 488 Carboxylic Acid, Succinimidyl Ester,**
Agg	**Aggregates of α-synuclein**
BiFC	**Bimolecular fluorescence complementation**
BSA	**Bovine serum albumin**
CRISPR/Cas9	**Clustered regularly interspaced short palindromic repeats /CRISPR-associated protein 9**
DMEM	**Dulbecco's Modified Eagle Medium**
DMSO	**Dimethyl sulfoxide**
E. coli	***Escherichia coli***
EIPA	**5-(n-ethyl-n-isopropyl)-amiloride**
FBS	**Fetal Bovine Serum**
GFP	**Green fluorescentprotein**
HSPG	**Heparan sulfate proteoglycan**
Int-Agg	**Intermediate-aggregates of α-synuclein**
IPTG	**Isopropyl β-D-thioglactopyranoside**
kDa	**Kilodalton**
LB	**Luria-Bertani medium**
LB	**Lewy body**

LN	**Lewy Neurite**
mono-RFP	**Monomeric red fluorescent protein**
MβCD	**Methyl-β-cyclodextrin**
PBS	**Phosphate buffered saline**
PD	**Parkinson's disease**
PMSF	**Phenylmethylsulfonyl fluoride**
Pre-Agg	**Pre-aggregates of α-synuclein**
S.D.	**Standard deviation**
SDS	**Sodium dodecyl sulfate**
SNCA	**Synuclein alpha**
SV2	**α-Synuclein-Venus2**
TB	**Trypan blue**
TDP43	**TAR DNA-binding protein 43**
TE	**Trypsin-EDTA**
TEM	**Transmission electron microscopy**
Th T	**Thioflavin T**
TM	**Transmembrane**
UCHL1	**Ubiquitin C-terminal hydrolase L1**
V1S	**Venus1-α-synuclein**

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder and can occur in a sporadic or a familiar form (Lotharius and Brundin, 2002). The patients of this disease show death of dopaminergic neurons which produce dopamine in the substantia nigra. This causes reduced concentration of dopamine in the striatum (Dauer et al., 2003; Obeso et al., 2010). Therefore treatment of the dopamine precursor, levodopa, is the main therapy for PD patients. Despite its long clinical success, levodopa has some problems in use, because it has side effects such as dyskinesias and cannot inhibit some of PD symptoms (Smith et al., 2012). Therefore, new mechanism-based approaches for treatment of PD are needed.

Many factors have been explored to be related to pathogenesis of PD (Lotharius and Brundin, 2002). The factors are misfolded α-synuclein, particular gene mutations (such as *SNCA*, and *UCHL1*), synthesis of dopamine-quinone species which are associated to oxidative stress and disrupted proteasomal degradation pathway. Molecular mechanism

underlying the pathogenesis of PD through these neuropathic factors is actively elucidated.

Lewy bodies (LB) and Lewy neurites (LN) in neurons are the histological hallmark of synucleinopathies, including Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy. LB and LN are mainly composed of α-synuclein (Snead and Eliezer, 2014). α-Synuclein is a soluble natively unfolded protein predominantly located in presynaptic region. Its oligomerization and fibrillation happen in PD brain. Although it is not clear which forms of α-synuclein are toxic, α-synuclein is considered to be important to the death of doparminergic neurons (Lotharius and Brundin, 2002). Point mutations of *SNCA*, the gene encoding α-synuclein (A53T, A30P, and E46K) and *SNCA* multiplications cause familial form of this disease (Chartier-Harlin et al., 2004; Krüger et al., 1998; Polymeropoulos et al., 1997; Zarranz et al., 2004). These facts support the importance of α-synuclein in PD.

Nowadays, many neurodegenerative disease proteins, including pathological forms of α-synuclein, tau, amyloid β, and TDP43 are thought to propagate from one neuron to another neuron. After the internalization of these prion-like disease proteins, these proteins play a role as seeds for fibrillation of normal protein in the recipient neuron (Brettschneider, 2015).

Spreading pattern of α-synuclein pathology in PD patient's brain has been generally known (Braak, 2003). In 2008, two articles reported host-to-graft propagation of Lewy-like pathology (Li et al., 2008; Kordower et al., 2008). Evidence from animal model experiments also suggested that pathological α-synuclein could propagate (Luk et al., 2012a; Luk et al., 2012b; Masuda-Suzukake et al., 2013). In this context, how α-synucleinis released from neurons and how it is internalized to cells are central to understanding the progression of synucleinopathies, but are still elusive. Receptor-mediated endocytosis, macropinocytosis, direct penetration, and exosomal transport are the suggested routes for α-synuclein internalization (Oueslati, 2014). Until now, heparan sulfate proteoglycan (HSPG) is the only reported molecule as mediator for internalization of both α-synuclein and tau (Holmes et al., 2013). Discovery of another receptor for α-synuclein are important.

Studies on propagation of disease proteins have grown in importance in understanding the disease progression over recent years, and it has long been a question of great interest whether these proteins are infectious like prion. Many studies in the field of propagation have focused on how α-synucleinis released from various perspectives (Alvarez-Erviti et al., 2011; Pan-Montojo et al., 2012; Poehler et al., 2014). On the other hand, studies

on internalization were relatively less diverse. Researches on internalization have mainly tended to focus on antibody therapy for α-synuclein clearance (Bae et al., 2012; Tran et al., 2014). In this study, I established and utilized cell-based assay to screen for α-synuclein receptors which mediate cellular uptake of α-synuclein. The main aim of this study is to isolate membrane protein(s) regulating cellular uptake of α-synuclein during cell-to-cell transmission in neuronal cells.

MATERIALS AND METHODS

Expression and purification of α-synuclein

Escherichia coli BL21 cells transformed with 6X His-tagged human α-synuclein which was kindly provided by Dr. Park, Sang-Hyun (Seoul National University, Korea), were cultured in LB medium with 100 μg/ml ampicillin at 37°C. At an A600 of 0.4-0.6, α-synuclein expression was induced with 1 mM isopropyl β-D-thioglactopyranoside (IPTG) at 28°Cfor 3 h. The pellet collected by centrifugation was resuspended with 30 ml His-binding buffer (20 mM Tris-Cl, pH 8.0, 0.5 M NaCl 20 mM Imidazole) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), then was sonicated for 90-120 min. After centrifugation, α-synuclein was purified by using Ni-NTA Agarose chromatography from the supernatant. The purity of α-synuclein was analyzed by coomassie blue staining and the concentration was determined by the Bradford assay. Also, by loading α-synuclein together with a series of known dilutions of bovine serum albumin (BSA), concentration of α-synuclein was doubly calculated. Purified α-synuclein was transferred to 10 kDa molecular weight cut off SnakeSkin Dialysis Tubing (Thermo Scientific) and dialyzed against phosphate buffered saline

(PBS) to remove imidazole.

In vitro fibrillation of α-synuclein

Purified α-synuclein (1-3 mg/ml) was incubated at 37°C with shaking for 14 days. The solution was sonicated briefly for preparation of fibril form of seeds and then further incubated under the same condition for 7 days.

Thioflavin T assay

Fibrillation of α-synuclein was examined by Thioflavin T assay. The reactions containing α-synuclein (20μM) were mixed with 25 μM thioflavin T in 50 mM glycine buffer in of volume of 200 μl in a 96-well plate. After shaking at 37℃ for 20 min, the signal was measured with a Varian Cary Eclipse fluorescence spectrophotometer at excitation of 450 nm and emission of 482 nm.

Transmission electron microscopy

Protein solution were placed on the Formvar-coated nickel grids (Polyscience, 300 meshes, 24928) for 1 min and negatively stained with 2% uranyl acetate for 1 min. After washed twice with distilled water, the

samples were incubated until dry. The sample was observed under JEOL JEM 1010 transmission electron microscope at 80 kV.

Labeling α-synuclein with Alexa Fluor488

α-Synuclein fibrils were labelled with Alexa Fluor® 488 Carboxylic Acid, Succinimidyl Ester, mixed isomers (Thermo Scientific, USA) by incubating 2 mg/ml α-synuclein fibrils and AF488 dye for 1 h at room temperature and dialyzed against PBS using SnakeSkin Dialysis Tubing (Thermo Scientific, USA).

Mammalian expression cDNA library

The cDNA expression library was prepared as following. The cDNAs that encode membrane protein containing single transmembrane (TM) and multi-TM domain were searched in the gene collection list of our laboratory and then purified by DNA preparation column (GeneAll, Korea) for transfection into mammalian cells.

Cell culture and DNA transfection

SH-SY5Y human neuroblastoma cells, HT22 mouse hippocampal cells,

and HeLa human cervical carcinoma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone) containing 10% (v/v) fetal bovine serum (FBS) (Hyclone), Gentamicin (GIBCO), and Penicillin Streptomycin (GIBCO) at 37℃ under an atmosphere of 5% CO2. SN4741 dopaminergic neuronal cells were cultured in DMEM with FBS, Gentamicine, Penicillin Streptomycin and 1% glucose at 33℃ under an atmosphere of 5% CO2 as previously described (Son et al., 1999). Stable cell lines, Venus1-α-synuclein (V1S) and α-synuclein-Venus2 (SV2) SH-SY5Y cells were gifted by Dr. Lee, Seung-Jae (Konkuk University, Korea) and cultured as described previously (Bae et al., 2014). DNA and sgRNA transfection were performed with the lipofector-pMAX tranftection reagent (APTABIO) according to the manufacturer's recommendations.

Construction of sgRNA CRISPR/Cas9

Constructs of sgRNAs were generated by CRISPR/Cas9 system as previously described (Shalem et al., 2014). After target sequences were selected on exons of STM1D9 and MTM7A8 (each with two targets), gRNA oligos were designed, then cloned by the standard-ligation method with lenti CRISPR-V2 vector.

Uptake assay ofAF488-α-synuclein by fluorescence spectroscopy

After 24 h from transfection, cells were incubated with 0.1 μM Alexa Fluor488 labelled α-synuclein fibrils for 5 h. In order to remove extracellular α-synuclein aggregates or extracellular signal from the culture, Trypsin-EDTA solution (TE) or trypan blue were employed. To remove extracellular α-synuclein fibrils, TE(0.05%) was added to cell culture for 1 min and then washed twice with DMEM containing 10% FBS. To quench the fluorescence from extracellular AF488-α-synuclein fibrils, 0.05% trypan blue in PBS was added to cell culture for 2 min and washed with DMEM containing 10% FBS as described previously (Dementhon et al., 2012). While extracellular or membrane-bound AF488-α-synuclein fibrils were quenched by trypan blue, the internalized AF488-α-synuclein fibrils inside the cells were protected from quenching.

Fluorescence Spectroscopy

Samples were visualized with fluorescence microscope (Olympus) or the IN Cell Analyzer 2000 (GE Healthcare) by following the instruction of the manufacturer. For confocal microscopy, cells were grown on a coverglass and fixed with 4% paraform aldehyde for 15 min and washed with PBS. Coverslips were incubated with Hoechst 33258 and placed with

mounting solution (Sigma). Samples were visualized with a confocal laser scanning microscope (CarlZeiss, LSM700).

Internalization Inhibition

Prior to addition of α-synuclein aggregates, cells were preincubated with 5-(n-ethyl-n-isopropyl)-amiloride (EIPA), methyl-β-cyclodextrin (MβCD), or hypertonic sucrose for 30 min and internalization of α-synuclein was examined under fluorescence microscope.

Statistical Analysis

P-values were calculated by using one-tailed Student's t-test. Error bars represent S.D.

RESULTS

In vitro preparation of fluorescent α-synuclein aggregates

To prepare α-synuclein aggregates *in vitro* (Figure 1B), α-synuclein was expressed in *E.coli* BL21 and purified using Ni-NTA Agarose resin. In order to examine the purity of purified α-synuclein, purified α-synuclein was separated by SDS-PAGE and then stained with coomassie blue (Figure 1B), showing that the purity of α-synuclein was good to prepare its aggregates.

In general, α-synuclein (1.0 to 3.0 mg/ml) was labelled with fluorescent dye, Alexa Flour 488, by incubation. The resulting α-synuclein was then incubated *in vitro* at 37°C with shaking for the indicated times to form aggregates, as described in the scheme of Figure 1A. In the middle of the process, α-synuclein aggregates were subjected to sonication and then further incubated with shaking.

To compare the forms of α-synuclein resulting from the incubation and sonication, α-synucleins were examined under transmission electron microscope (TEM) (Figure 1C). Before the incubation, short form of α-synuclein aggregates (Pre-Agg) was observed under TEM. After 7 days with

shaking, longer form of protein aggregates was observed (Int-Agg). After sonication and further *in vitro* incubation, much longer form of protein aggregates were evidently observed (Agg). The amounts of protein aggregates increased proportionally to the incubation period. Further, using Thioflavin T which incorporates into β-sheets of protein aggregates and generates fluorescence at 482 nm, the difference in the aggregation between Pre-Agg and Agg was also confirmed (Figure 1D).

Establishment of α-synuclein uptake assay in neuronal cells

To establish an assay for α-synuclein internalization, internalized α-synuclein should be distinguished from extracellular α-synuclein. For this purpose, I employed two different approaches, brief digestion with Trypsin-EDTA (TE) and quenching with trypan blue (TB). As expected, treatment with TE or addition of TB reduced the fluorescence of AF488-labelled α-synuclein aggregates *in vitro* (Figure 2A). In these *in vitro* experiments, the concentration of α-synuclein analyzed was much higher (5 μM) than that of cellular assay (0.1 μM), while the concentrations of TE and TB were same as cellular assay. Under this *in vitro* assay, TB seems to be better to reduce the fluorescent signal of extracellular AF488-α-synuclein.

Then, I also tested these two methods in cellular uptake of α-synuclein. When SH-SY5Y cells were incubated with AF488-labelled α-synuclein aggregates, I observed intracellular fluorescence (*arrowheads*) and extracellular AF488-α-synuclein aggregates (*arrows*) (Figure 2B, control). Even after washing with PBS, extracellular fluorescence of AF488-α-synuclein aggregates were still observed together with intracellular fluorescence (Figure 2B, PBS). Consistent to the results of *in vitro* experiments, however, treatment with TE or TB efficiently reduced extracellular fluorescence of AF488-α-synuclein, leaving the fluorescence inside of cells (Figure 2B, TE and TB). These results suggest that TE or TB can be applied for high-contents screening to reduce the noise of cellular uptake assay.

Neuronal uptakeof α-synuclein fibrils increases in a time and dose-dependent manner

Next, I characterized cellular uptake of α-synuclein in SH-SY5Y cells. Alexa Fluor 488 dye alone was not internalized into SH-SY5Y cells (Figure 3A). When SH-SY5Y cells were incubated with different forms of α-synuclein, Int-agg and Agg, aggregated form of α-synuclein (Agg) was

internalized better than less-aggregated form of α-synuclein (Int-Agg) (Figure 3A and 3B). Also, cellular uptake of α-synuclein into SH-SY5Y cells was increased when SH-SY5Y cells were incubated with increasing concentrations of α-synuclein (Figure 3C) and with α-synuclein for longer times (Figure 3D), indicating that cellular uptake of α-synuclein increases in a dose- and time-dependent manner.

In addition, cellular uptake of with α-synuclein was also examined under confocal microscope. SH-SY5Y cells were transfected with mono-RFP and then incubated with AF488-α-synuclein. After staining nuclei with Hoechst 33258, the results from fluorescence confocal microscopy showed that AF488-α-synuclein apparently existed in SH-SY5Y cells (Figure 3E). Together, these results suggest that there is a cellular uptake system in SH-SY5Y cells.

Characterization of cellular uptake pathways of α-synuclein in SH-SY5Y cells

To gain insight into how cells internalize α-synuclein, I analyzed uptake pathways using pathway inhibitors. Treatment with 5-(n-ethyl-n-isopropyl)-amiloride (EIPA), an inhibitor of macropinocytosis, or sucrose (hypertonic),

an inhibitor of clathrin-mediated endocytosis, blocked cellular uptake of α-synuclein (Figure 4A and 4B). On the contrary, MβCD, a blocker of caveolin-mediated endocytosis, did not affect it. Consistently with a recent report that tau is internalized via macropinocytosis (Holmes et al., 2013), these results suggest that α-synuclein may be internalized via utilizing plasma membrane proteins as receptors.

Functional screening and isolation of membrane proteins stimulating cellular uptake of α-synuclein

Then I screened membrane proteins using this cell-based assay and cDNAs expression library. From cDNA expression library in our laboratory, I collected total 1,500 cDNAs encoding membrane proteins harboring 496 single transmembrane (TM) and 1,036 multi-TM domains. To identify the transfected cells with cDNA, mono-RFP was included in every transfection. In the primary screening, SH-SY5Y cells were transiently co-transfected with mono-RFP and each cDNA in single TM pool (stock plate # 1-6) or multi TM pool (stock plate # 1-12). After incubation with AF488-α-synuclein, the reaction was quenched by adding TB.

Analysis of α-synuclein uptake was then analyzed with high-contents

screening using image program of IN Cell Analyzer 2000. Because there was a positive correlation between α-synuclein uptake and rather its concentration (r = 0.88) or exposure time (r = 0.69), single concentration (0.1 μM) and incubation time (5 h) were used for the screening and analysis. From the primary screening, I selected two to ten putative positive clones per stock plate (96 well) (Figure 5A and 5B). Total 50 putative clones were again tested for their ability to affect α-synuclein uptake using the same assay. From the secondary screening of those putative clones, nine clones were finally isolated to stimulate α-synuclein uptake as much as positive control in neuronal cells (Figure 6A). Most of them have reported function in diverse pathways, including neuronal transport and are briefly summarized in Table 1.

STM1D9 and MTM7A8 stimulate cellular uptake of α-synuclein aggregates, but not amyloid β and tau aggregates

Among those candidate clones, STM1D9 and MTM7A8 were most effective to stimulate cellular uptake of α-synuclein aggregates. STM1D9 has single TM and MTM7A8 harbors multi-TM. Thus, I characterized their activities in detail. In particular, there was a need to confirm their effects

again by using TE assay and compare it with TB assay. As can be seen from Figure 7A and 7B, STM1D9 and MTM7A8 were still effective to stimulate cellular uptake of α-synuclein aggregates in TE assay but MTM7A8 was less effective than TB to stimulate cellular uptake of α-synuclein aggregates.

Again, it was also interesting to characterize their effect on the internalization of different forms of α-synuclein aggregates, such as Pre-Agg, Int-Agg, and Agg. Overexpression of STM1D9 and MTM7A8 increased cellular uptake of Int-Agg and Agg α-synuclein aggregates but not Pre-Agg (Figure 7C). In this experiment, SDC1, one of the HSPGs' core protein, which was recently reported (Holmes et al., 2013), as a receptor of tau and α-synuclein was served as a positive control.

Next, to examine knockout effect of STM1D9 and MTM7A8 on cellular uptake of α-synuclein aggregates, sgRNA using CRISPR/Cas9 technology was employed. Two sgRNA for each STM1D9 and MTM7A8 were constructed. Compared to control cells, transient transfection with either sgSTM1D9#1 or #2 reduced cellular uptake of α-synuclein in SH-SY5Y cells. In addition, sgMTM7A8 #1 and #2 also reduced α-synuclein internalization. Together with their overexpression effects, these results suggest that STM1D9 and MTM7A8 function in cellular uptake of α-synuclein in SH-SY5Y cells.

Like α-synuclein, tau and amyloid β have also received considerable attention for their characteristics of cell-to-cell transmission. Thus, the impact of STM1D9 and MTM7A8 on cellular uptake of tau aggregates and amyloid β oligomers was examined. However, ectopic expression of STM1D9 and MTM7A8 did not stimulate cellular uptake of tau and amyloid β in SH-SY5Y cells (Figure 8A and 8B).

STM1D9 and MTM7A8 stimulate cellular uptake of α-synuclein in other cells

In SN4741 mouse dopaminergic neuronal cells, HT22 mouse hippocampal cells, and HeLa human cervical carcinoma cells, overexpression of STM1D9 and MTM7A8also increases α-synuclein internalization (Figure 9A-B; Table 2).

Overexpression of STM1D9 or MTM7A8 increases cell-to-cell transmission of α-synuclein aggregates

More importantly, I addressed whether STM1D9 and MTM7A8 could affect cell-to-cell transmission of α-synuclein in neuronal cells. To address

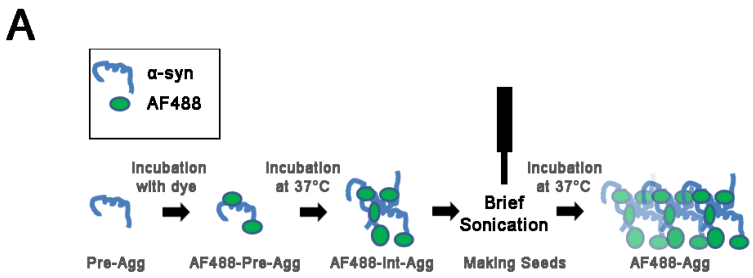
this question, bimolecular fluorescence complementation (BiFC) assay was employed, which allows to observe fluorescence only under protein-protein interaction in live cells. This dual-cell BiFC system was developed as a cell-based method to study cell-to-cell transmission of α-synuclein (Bae et al., 2014). The α-synuclein was fused to either the N- or C-terminal half of GFP to generate Venus1-α-synuclein (V1S) and α-synuclein-Venus2 (SV2), respectively. Then, stable SH-SY5Y cells expressing either V1S or SV2 were generated. When co-cultured, cells can show BiFC fluorescence only through formation of V1S/SV2 aggregates in the same cells, resulting from cell-to-cell transmission of α-synuclein.

When the BiFC cells (V1S and SV2) were co-cultured, there was a very few signals. The signals were appeared as shown in Figure 10A. There was a need to increase the fluorescence in the BiFC assay because the percentage of cells showing the BiFC fluorescence was very low (<1%) in this culture condition. By adding α-synuclein aggregates (*in vitro* prepared, unlabelled) as a seed, I found that the overall BiFC intensity increased (Figure 10A and 10B, see pcDNA3-transfected groups in 10B). Even without treatment of α-synuclein aggregates as a seed, transfection with STM1D9 or MTM7A8 significantly increased the number of cells showing BiFC (Figure 10B: light gray bars). In addition, here as in elsewhere, transfection with STM1D9 or

MTM7A8 also increased the BiFC fluorescence in the presence of α-synuclein aggregates. These results indicate that STM1D9 and MTM7A8 might also function in cell-to-cell transmission of α-synuclein aggregates.

Figure 1 | Preparation and characterization of α-synuclein aggregates.

(A) *In vitro* fibrillation of α-synuclein was performed according to the schematic diagram. Prior to undertaking fibrillation, purified α-synuclein (Pre-Agg) was labelled with Alexa Fluor-488 (AF488) dye if necessary. AF488-labelled or unlabelled α-synuclein was incubated at 37°C for 7 days with shaking (Int-Agg). The protein was further incubated for 14 days under the same condition, and at the 7th day the protein was sonicated once (Agg). (B) His-α-synuclein was expressed in *E. coli* BL21 cells and purified by using Ni-NTA Agarose resin. After separating the protein by SDS-PAGE, the purity of the purified protein was analyzed by coomassie blue staining method. (C) Transmission electron microscope images of α-synuclein at different stages of aggregation. Scale bar = 0.5 μm. (D) Thioflavin T assay showing the amyloidosis of α-synuclein. The α-synuclein at different stages of aggregation was incubated with thioflavin T and the fluorescence intensity was measured at 482 nm. Bars represent standard deviation. $^{**}P<0.001$.


A
α-syn
AF488
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Incubation at 37°C
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Making Seeds
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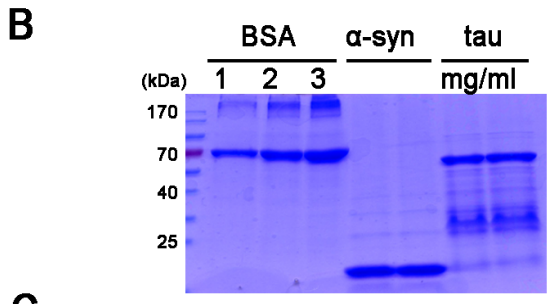
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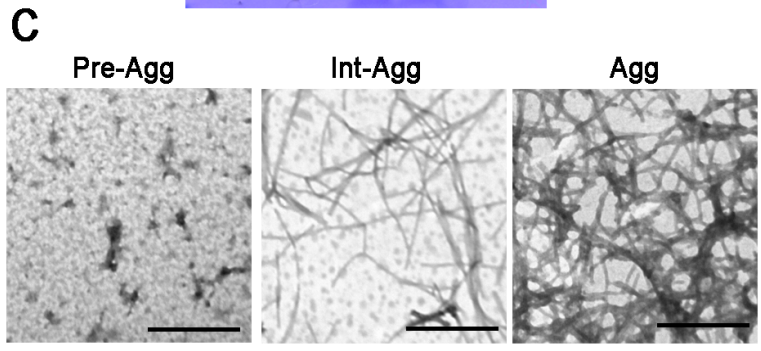
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Int-Agg
Agg

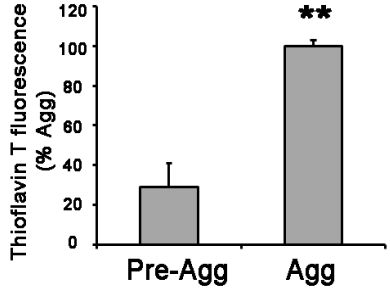
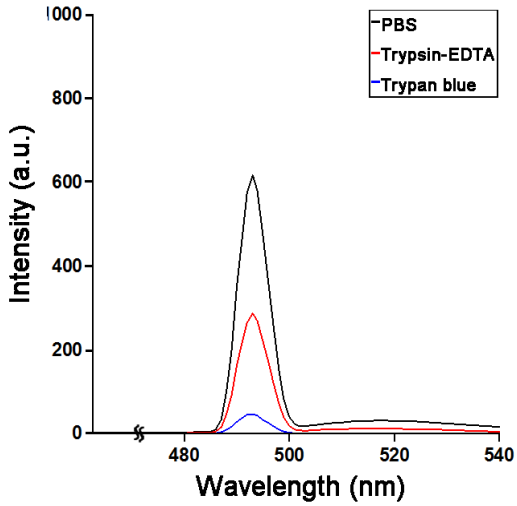

D
Thioflavin T fluorescence (% Agg)
120
100
80
60
40
20
0
**
Pre-Agg
Agg

Figure 2 │Assay development for cellular uptake of extracellular α-synuclein

(A) AF488-labelled aggregates (AF488-Agg, 5 μM) were left untreated (PBS) or treated with either Trypsin-EDTA (TE, 0.05%) or trypan blue (TB, 0.05%) *in vitro*. By using fluorescence spectrophotometer, emission spectrum was then analyzed. (B) SH-SY5Y cells were incubated with 0.1 μM AF488-Agg for 5 h. Cells were left untreated, or treated with PBS for washing, or Trypsin-EDTA (0.05%) at 37°C for 1 min, or trypan blue (0.05%) for 2 min. Following the treatments, cells were washed with cell culture medium again and observed under fluorescence microscope. *Arrowheads* indicate extracellular α-synuclein and *arrows* indicate intracellular α-synuclein. Scale bar = 50 μm.

A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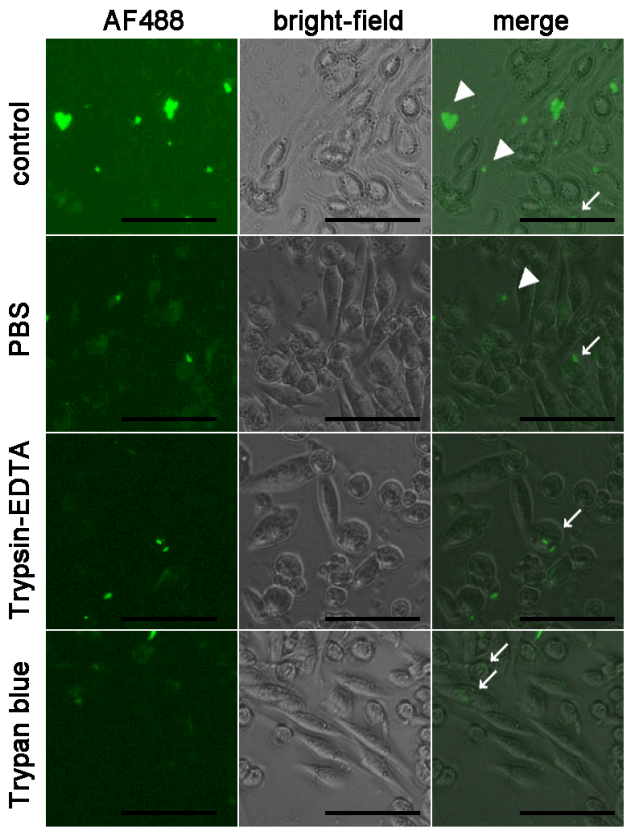
Wavelength (nm)

B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bright-field
merge
control
PBS
Trypsin-EDTA
Trypan blue

Figure 3 │The internalization of α-synuclein into SH-SY5Y cells.

(A) SH-SY5Y cells were transfected with mono-RFP for 24 h, incubated with different forms of 0.1 μM α-synuclein, Pre-Agg, Int-Agg, and Agg, for 5 h and then observed under fluorescence microscope. Scale bar = 50 μm. (B) Quantification of the images in (A). Percentages of cells with α-synuclein were determined. Bars represent mean values ± standard deviation. (C) Dose-dependent internalization of α-synuclein. SH-SY5Y cells were incubated with the increasing concentrations of α-synuclein (AF488-Agg). Percentage of cells with AF488-Agg was determined. Bars represent mean values ± standard deviation. (D) Time-dependent internalization of α-synuclein. SH-SY5Y cells were incubated with0.1 μM α-synuclein (AF488-Agg) for the indicated times. Percentage of cells with AF488-Agg were determined. Bars represent mean values ± standard deviation. (E) Cellular location of internalized α-synuclein. SH-SY5Y cells were transfected with mono-RFP for 24 h and then incubated with 0.1 μM α-synuclein (AF488-Agg) for 5 h. After staining with Hoechst 33258, cells were examined by confocal microscopy. Scale bar = 20 μm.

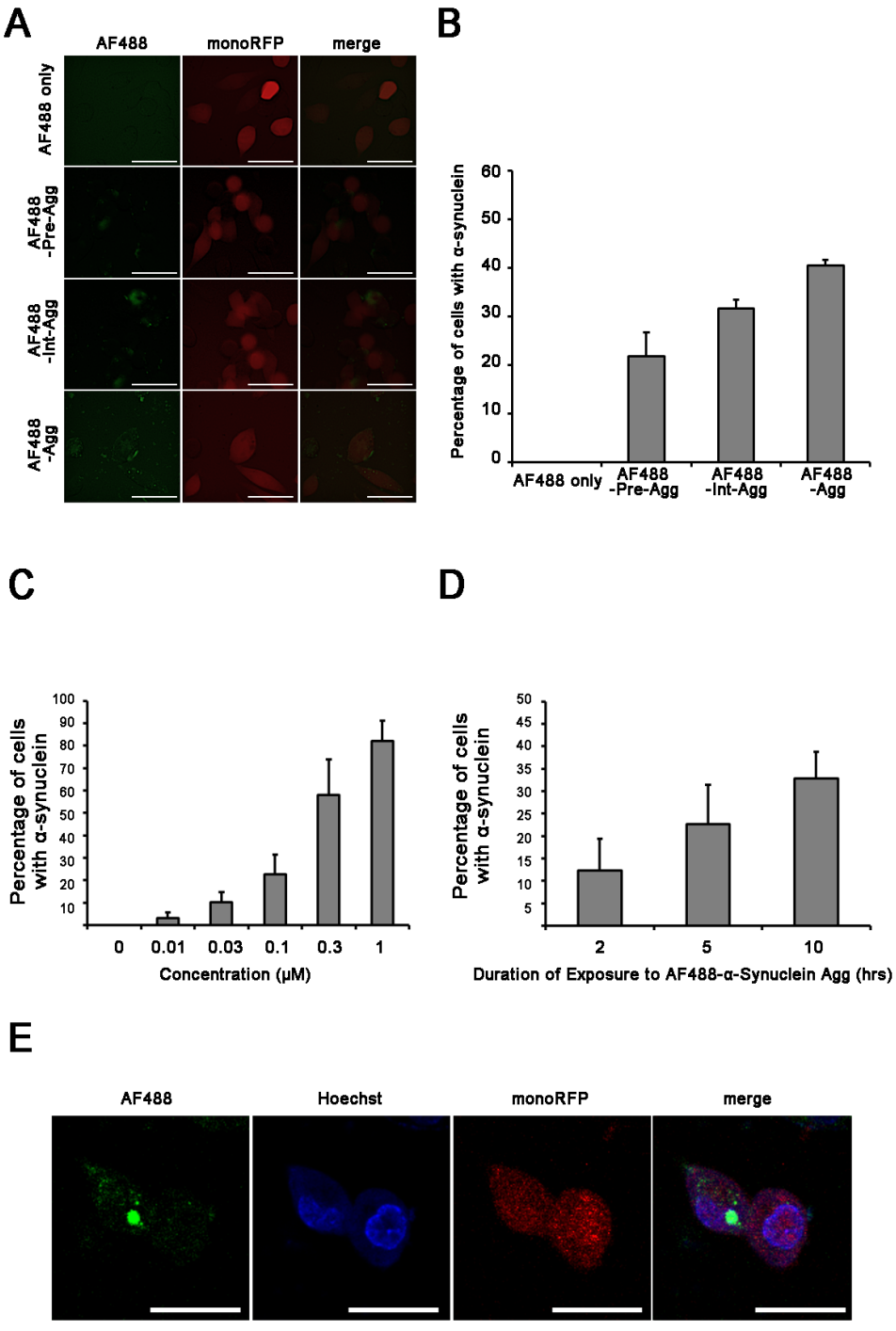

A
AF488
monoRFP
merge
AF488 only
AF488 -Pre-Agg
AF488 -Int-Agg
AF488 -Agg
B
Percentage of cells with α-synuclein
60
50
40
30
20
10
0
AF488 only
AF488 -Pre-Agg
AF488 -Int-Agg
AF488 -Agg
C
Percentage of cells with α-synuclein
100
90
80
70
60
50
40
30
20
10
0
0.01
0.03
0.1
0.3
1
Concentration (μM)
D
Percentage of cells with α-synuclein
50
45
40
35
30
25
20
15
10
5
2
5
10
Duration of Exposure to AF488-α-Synuclein Agg (hrs)
E
AF488
Hoechst
monoRFP
merge

Figure 4 │Effects of endocytosis inhibitors on the internalization of α-synuclein into SH-SY5Y cells.

(A) SH-SY5Y cells were pretreated for 30 min with cell culture medium containing dimethyl sulfoxide (DMSO), 50 μM 5-(n-ethyl-n-isopropyl)-amiloride (EIPA), 0.5 mM methyl-β-cyclodextrin (MβCD) or 0.2 M sucrose (hypertonic). These drug-treated cells were subsequently exposed to 0.1 μM AF488-Agg for 5 h and treated with trypan blue. Cells were observed under fluorescence microscope. Scale bar = 500 μm. (B) Quantification of the images in (A). Percentages of cells with α-synuclein were determined. Bars represent mean values ± standard deviation. $^{**}P < 0.01$.

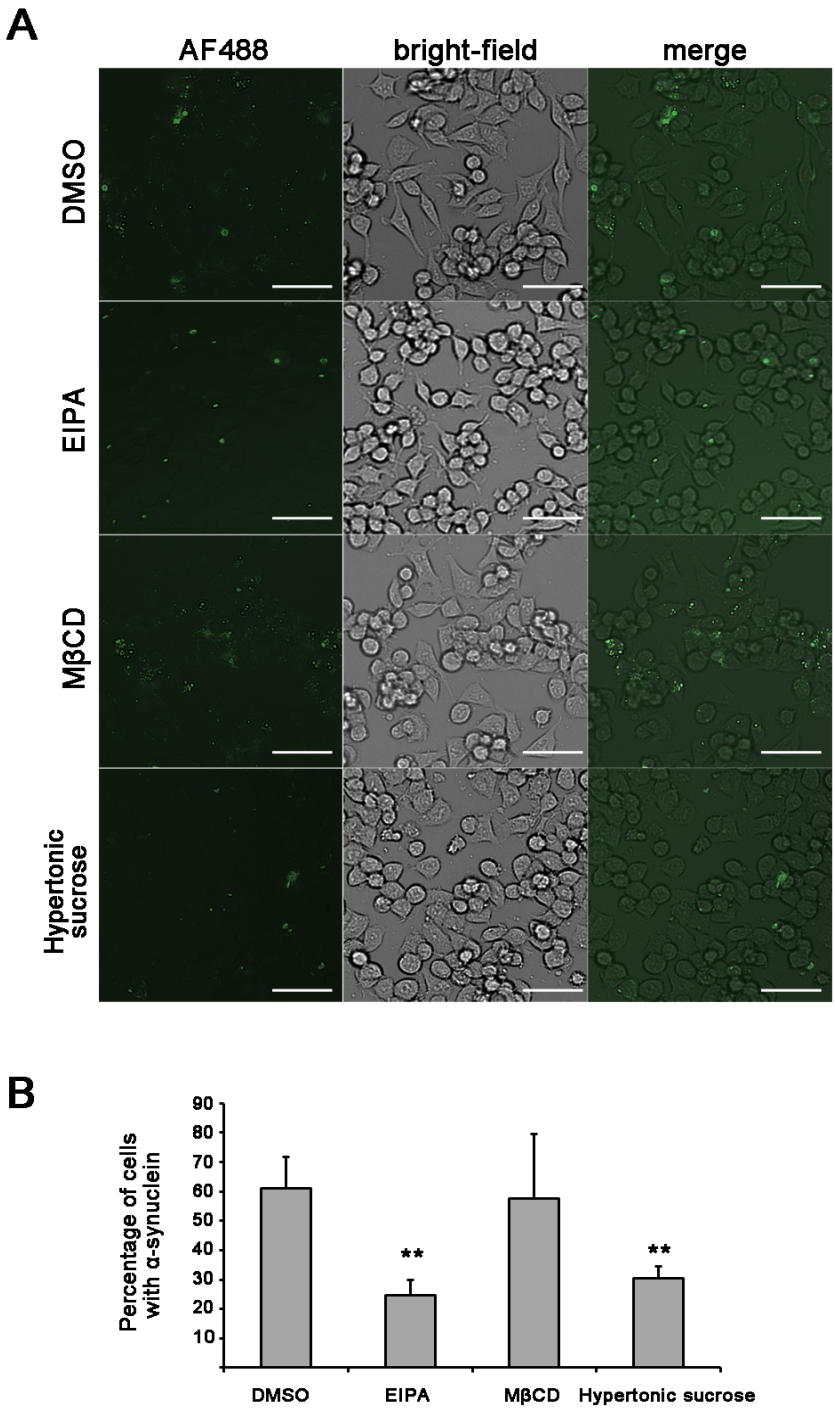

A
AF488
bright-field
merge
DMSO
EIPA
MβCD
Hypertonic sucrose
B
Percentage of cells with α-synuclein
90
80
70
60
50
40
30
20
10
**
**
DMSO
EIPA
MβCD
Hypertonic sucrose

Figure 5 │Primary screenings of membrane proteins mediating α-synuclein internalization.

(A, B) Primary high-contents screening. SH-SY5Y cells were subcultured onto 96-well tissue culture plate one day before and then co-transfected with mono-RFP and either controls or each cDNA encoding single TM-containing membrane protein (STM, plate # 1-6, total 496 genes) (A) or multi-TM-containing membrane protein (MTM, plate # 1-12, total 1036 genes) (B). After 24 h, cells were incubated with 0.1 μM AF488-Agg for 5 h and treated with trypan blue. Cells were then analyzed using IN Cell analyzer 2000.

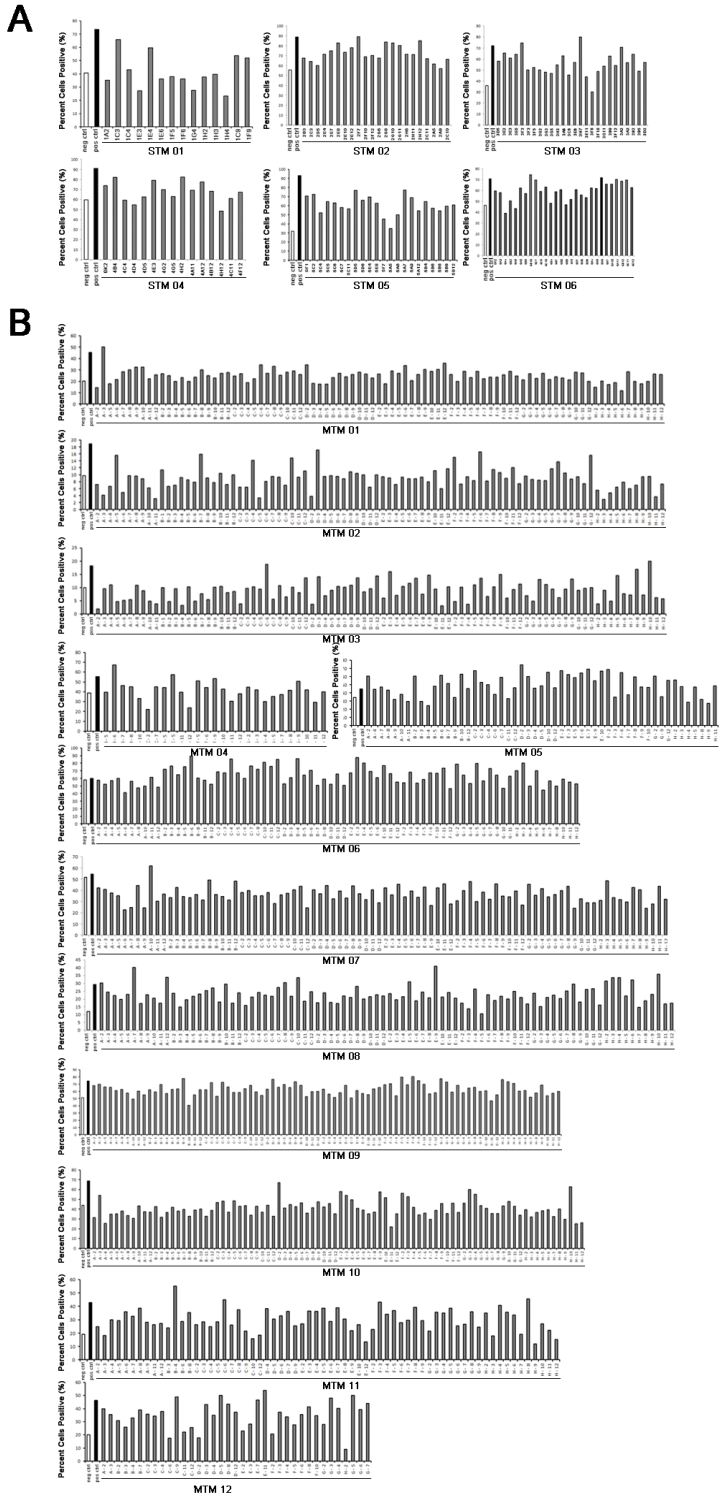

A
Percent Cells Positive (%)
STM 01
STM 02
STM 03
STM 04
STM 05
STM 06
B
MTM 01
MTM 02
MTM 03
MTM 04
MTM 05
MTM 06
MTM 07
MTM 08
MTM 09
MTM 10
MTM 11
MTM 12

Figure 6 │Secondary screening of membrane protein stimulating α-synuclein internalization.

SH-SY5Y cells were subcultured onto 96-well tissue culture plate one day before and then co-transfected with mono-RFP and either controls or one of the putative positive clones (two to ten putative positive clones per stock plate for primary screening). After 24 h, cells were incubated with 0.1 μM AF488-Agg for 5 h and treated with Trypan blue. Cells were then analyzed using IN Cell analyzer 2000. The putative positive candidates from primary screening were tested again. Bars represent mean values ± standard deviation. **P*< 0.05. ***P*< 0.01.

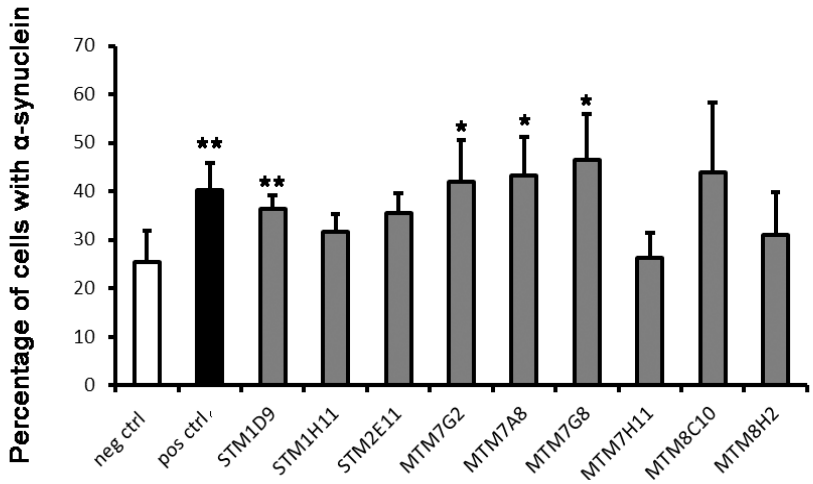

Percentage of cells with α-synuclein
70
60
50
40
30
20
10
0
**
**
*
*
*
neg ctrl
pos ctrl
STM1D9
STM1H11
STM2E11
MTM7G2
MTM7A8
MTM7G8
MTM7H11
MTM8C10
MTM8H2

Figure 7 │STM1D9 and MTM7A8 stimulate cellular-uptake of α-synuclein in SH-SY5Y cells.

(A) SH-SY5Y cells were transfected with mono-RFP and pcDNA3 (negative control), SDC1 (positive control), STM1D9 or MTM7A8 for 24 h, incubated with different forms of 0.1 μM α-synuclein aggregates (AF488-Agg) for 5 h. After addition with Trypsin-EDTA or Trypan blue, cells were observed under fluorescence microscope. Scale bar = 50 μm. (B) Quantification of the images in (A). Percentages of cells with α-synuclein were determined. Bars represent mean values ± standard deviation. (C) SH-SY5Y cells were transfected with mono-RFP and pcDNA3 (negative control), SDC1 (positive control), STM1D9 or MTM7A8 for 24 h, incubated with different forms of 0.1 μM α-synuclein for 5 h. Cells were then incubated with AF488-Pre-Agg, AF488-Int-Agg, or AF488-Agg α-synuclein. Cells were analyzed under IN Cell Analyzer 2000. Percentages of cells with α-synuclein were determined. (D) SH-SY5Y cells were transfected with control sgRNA (Cas9/CRISPR), sgSTM1D9 (#1 and 2), or sgMTM7A8 (#1 and 2) for 2 days and then treated with 0.1 μM AF488-Agg for 5 h, then treated with trypan blue. Bars represent mean values ± standard deviation.

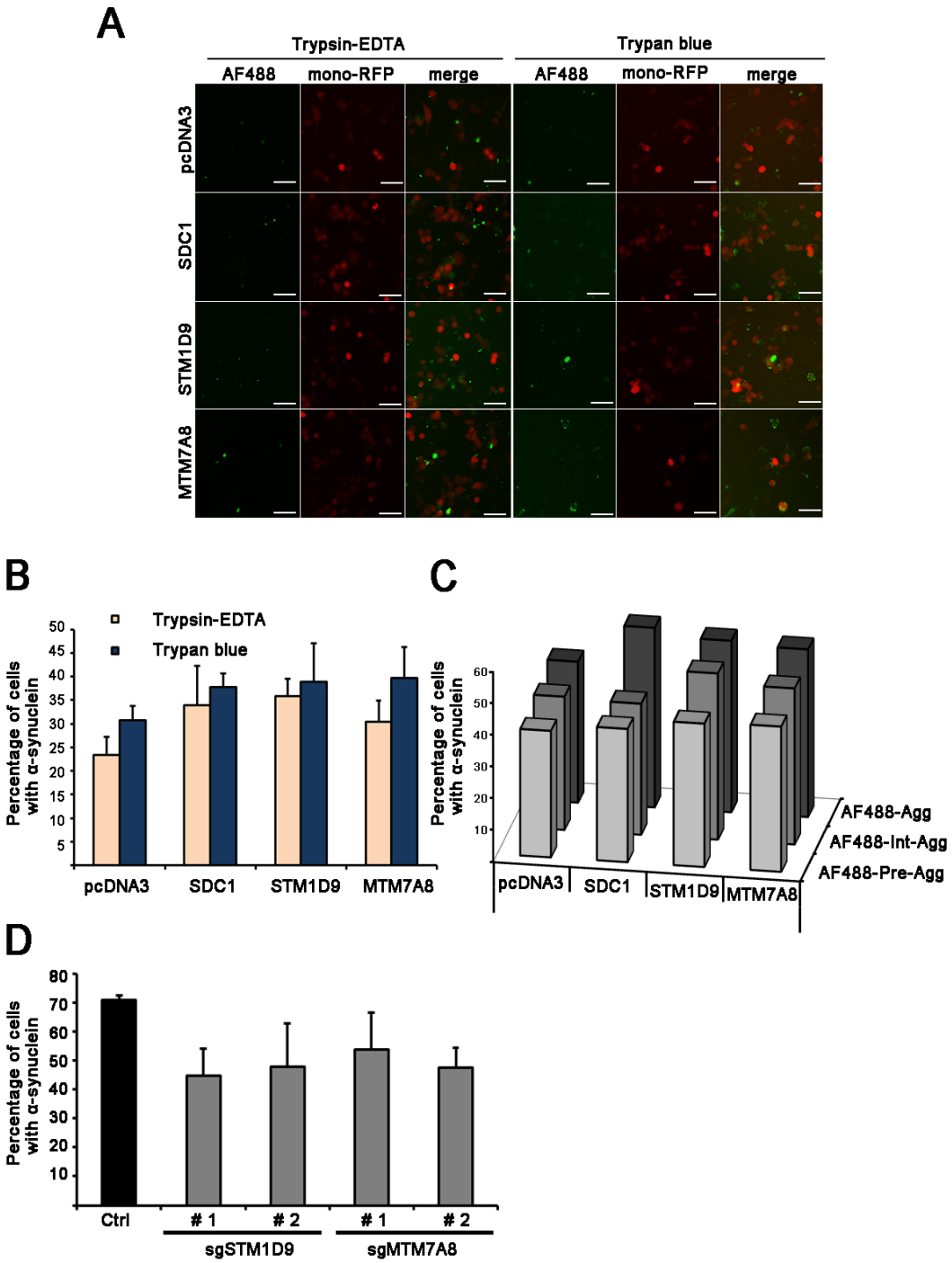

A
Trypsin-EDTA
Trypan blue
AF488
mono-RFP
merge
pcDNA3
SDC1
STM1D9
MTM7A8
B
Trypsin-EDTA
Trypan blue
Percentage of cells with α-synuclein
pcDNA3
SDC1
STM1D9
MTM7A8
C
AF488-Agg
AF488-Int-Agg
AF488-Pre-Agg
D
Ctrl
1
2
sgSTM1D9
sgMTM7A8

Figure 8 │ Effects of STM1D9 or MTM7A8 overexpression on tau and Aβ internalization.

(A) SH-SY5Y cells were co-transfected with mono-RFP, and either pcDNA3, STM1D9 or MTM7A8. After 24 h, cells were incubated with 0.5 μM AF488-labelled tau aggregates for 5 h and then analyzed by IN Cell Analyzer 2000. Scale bar = 50μm. (B) Quantification of the images in (A). Percentages of cells with tau aggregates were determined. Bars represent mean values ± standard deviation. (C) SH-SY5Y cells were co-transfected with mono-RFP, and either pcDNA3, STM1D9 or MTM7A8. After 24 h, cells were incubated with 125 nM FITC-Aβ oligomers for 2 h. Scale bar = 50 μm. (D) Quantification of the images in (C). Percentages of cells with FITC-Aβ oligomers were determined. Bars represent mean values ± standard deviation.

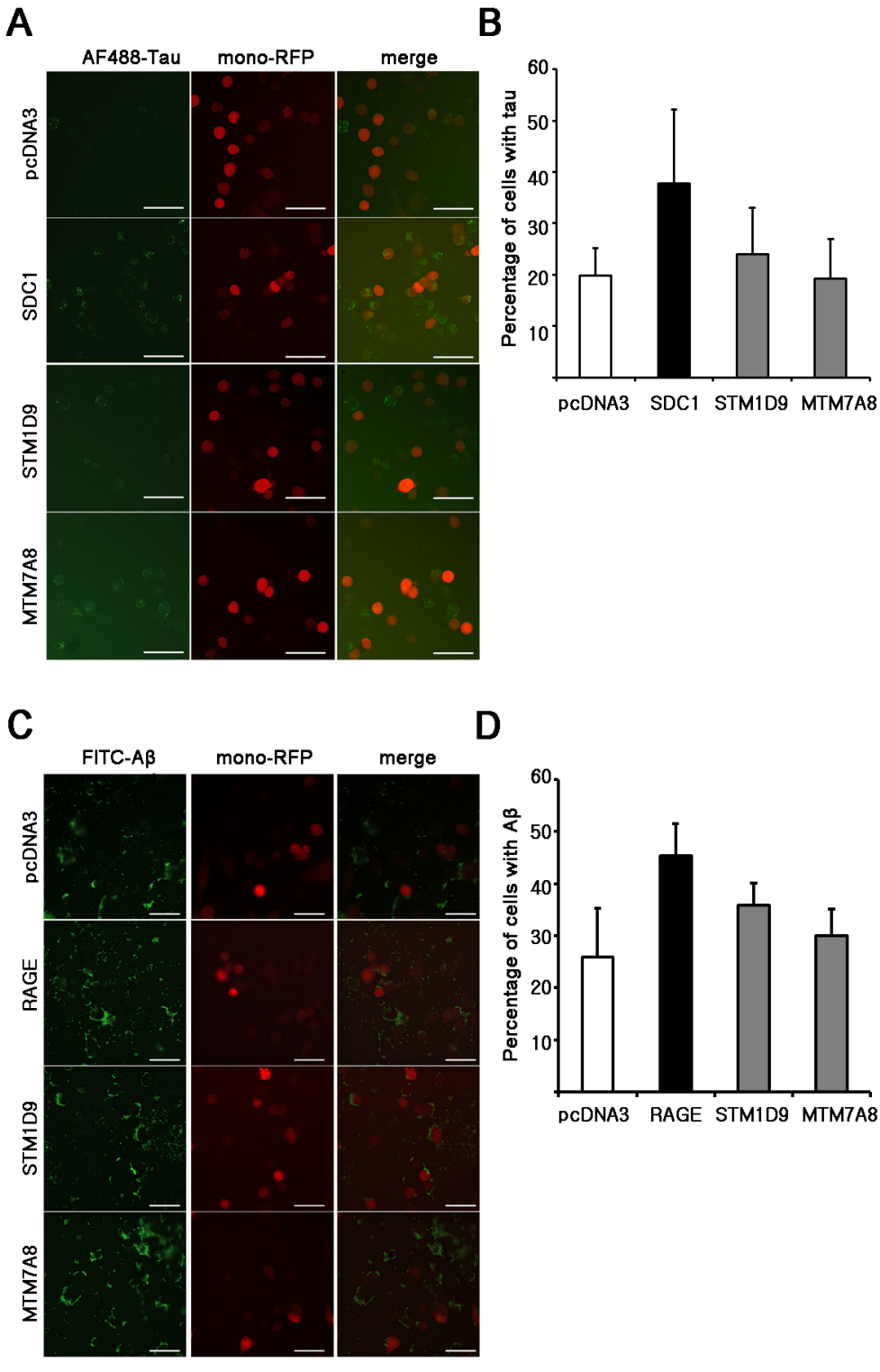

A
AF488-Tau
mono-RFP
merge
pcDNA3
SDC1
STM1D9
MTM7A8
B
Percentage of cells with tau
60
50
40
30
20
10
pcDNA3
SDC1
STM1D9
MTM7A8
C
FITC-Aβ
mono-RFP
merge
pcDNA3
RAGE
STM1D9
MTM7A8
D
Percentage of cells with Aβ
60
50
40
30
20
10
pcDNA3
RAGE
STM1D9
MTM7A8

Figure 9 | Internalization of α-synuclein in SN4741 dopaminergic neuronal cells.

(A) SN4741 dopaminergic neuronal cells were co-transfected with mono-RFP, and either pcDNA3, STM1D9 or MTM7A8. After 24 h, cells were incubated with 0.1μM AF488-labelled α-synuclein aggregates (AF488-Agg) for 5 h and then analyzed by IN Cell Analyzer 2000. Scale bar = 50μm. (B) Quantification of the images in (A). Percentages of cells with α-synuclein aggregates were determined. Bars represent mean values ± standard deviation. *$P< 0.05$. **$P< 0.01$.

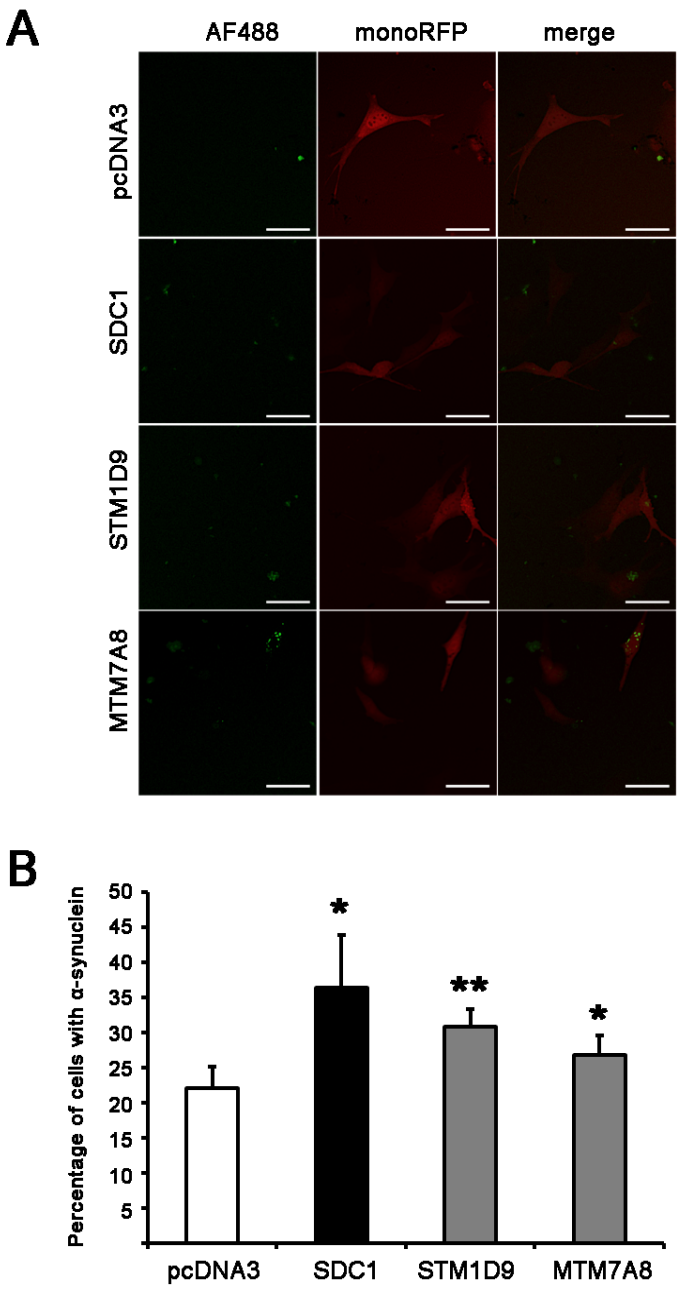
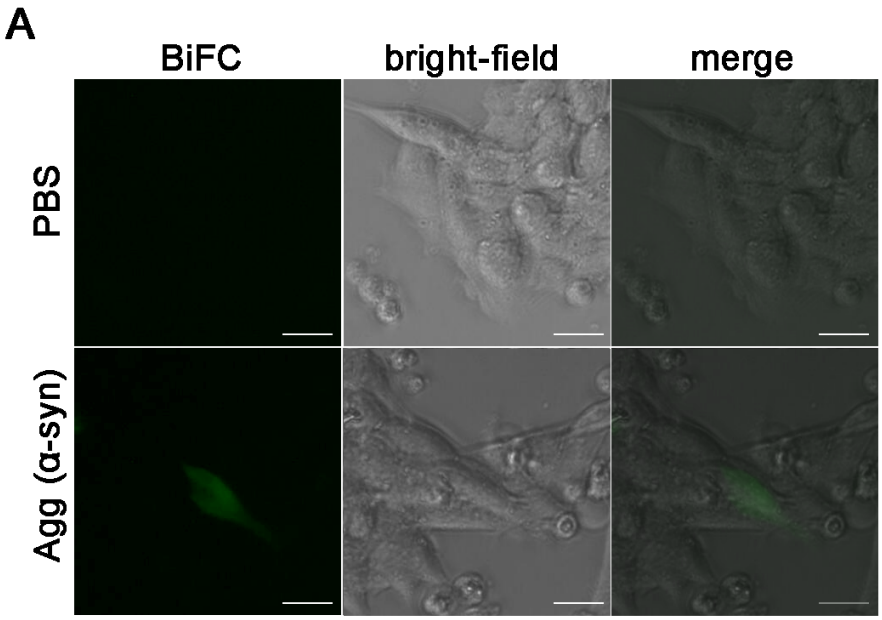

A
AF488
monoRFP
merge
pcDNA3
SDC1
STM1D9
MTM7A8
B
Percentage of cells with α-synuclein
50
45
40
35
30
25
20
15
10
5
*
**
*
pcDNA3
SDC1
STM1D9
MTM7A8

Figure 10 │ Stimulatory effects of STM1D9 and MTM7A8 on cell-to-cell transmission of α-synuclein in BiFC system.

(A) Typical image showing cell-to-cell transmission of α-synuclein in BiFC assay. SH-SY5Y cells expressing the Venus N-terminal fragment conjugated to human α-synuclein (VS1), and SH-SY5Y cells expressing the Venus C-terminal fragment conjugated human α-synuclein (SV2) were co-cultured for 30 h and were treated with PBS or unlabelled α-synuclein aggregates for 24 h. Then, SH-SY5Y cells showing the fluorescence from BiFC signals were observed under fluorescence microscope. (B) STM1D9 and MTM7A8 stimulate cell-to-cell transmission of α-synuclein in BiFC assay. VS1/VS2 cells were transfected with pcDNA3, SDC1, STM1D9 or MTM7A8 for 24 h and after 6 h, cells were added with α-synuclein aggregates for 24 h. The numbers of SH-SY5Y cell showing the fluorescence were then counted under fluorescence microscope. Bars represent mean values ± standard deviation. $*P< 0.05$. $**P< 0.01$. (when compared to pcDNA3.)


A
BiFC
bright-field
merge
PBS
Agg (α-syn)

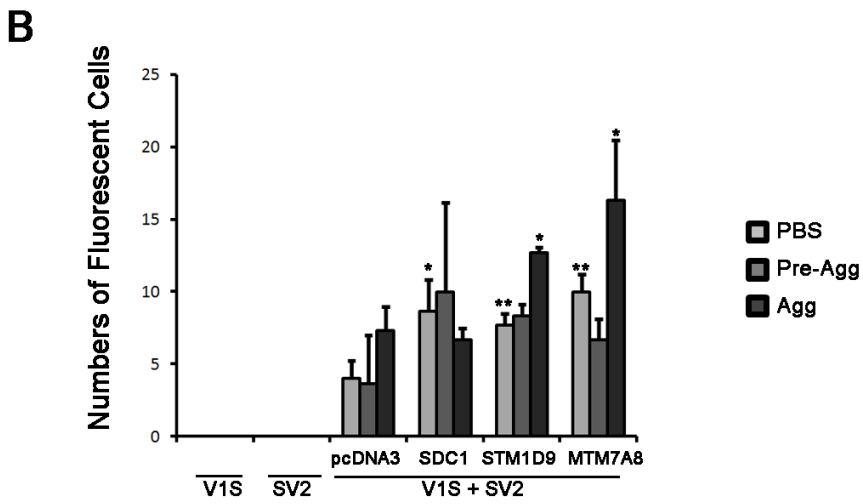

B
Numbers of Fluorescent Cells
25
20
15
10
5
0
pcDNA3
SDC1
STM1D9
MTM7A8
V1S
SV2
V1S + SV2
PBS
Pre-Agg
Agg

Table 1 │List and proposed function of the putative positive clones after the secondary screening.

Nine putative candidate genes isolated to stimulate cellular uptake of α-synuclein aggregates were investigated.

name	summary
STM1D9	a kind of glycan, expressed in the cerebrovascular system
STM1H11	related to granulation and redistribution of plasma membrane
STM2E11	uncharacterized
MTM7G2	unknown (function), potentially related to Down syndrome congenital heart disease
MTM7A8	known as a transporter protein for certain neurotransmitter, in central nervous system
MTM7G8	located to early endosomes, a potent ion channel or transporter for small molecules
MTM7H11	known as a nuclear-encoded transporters
MTM8C10	related to flow of cholesterol into mitochondria
MTM8H2	G-protein coupled receptors

Table 2 ｜Internalization of α-synuclein in other cell lines.

SH-SY5Y cells, HT22 cells, and HeLa cells were co-transfected with mono-RFP, and either pcDNA3, STM1D9 or MTM7A8. After 24 h, cells were incubated with 0.1μM AF488-labelled α-synuclein aggregates (AF488-Agg) for 5 h and then observed under fluorescence microscope. The more plus signs (+) are presented, the more cells harbor α-synuclein aggregates.

	neg ctrl	pos ctrl	STM1D9	MTM7A8
SH-SY5Y	·	+++	++	++
HT22	·	+++	+	++
HeLa	+	+++	++	+++

DISCUSSION

In this study, an assay for the screening of α-synuclein internalization stimulators was successfully established. Especially, oligomeric forms or aggregates of α-synuclein are a species to be internalized into neuronal cells. Utilizing the assay, STM1D9 and MTM7A8 were selected as putative candidates of membrane proteins regulating cellular uptake of α-synuclein aggregates. Apparently, expression level of STM1D9 and MTM7A8 affected cellular uptake of α-synuclein aggregates. These clones also seem to function to mediate cell-to-cell transmission of α-synuclein in neuronal cells as assessed by the BiFC system. Compared to loss-of-functional screening utilizing siRNA library, the advantage of gain-of-functional screening using cDNA expression library is to make it possible to find out the signaling regulator otherwise not working in culture cells.

Although one major issue in synucleinopathies is cell-to-cell transmission of α-synuclein, the molecules which mediate internalization of this protein are not known. Recently, HSPG was reported to mediate cellular uptake of tau protein and α-synuclein (Holmes et al., 2013). HSPG is a large

and sticky glycosaminoglycan and typically makes complexes with co-receptor proteins when it interacts with other proteins. When I tested the activity of SDC1, one of the HSPGs' core protein, for cellular uptake of α-synuclein aggregates, I found that it could also function to internalize α-synuclein aggregates. One of the selected candidates, STM1D9, is also glycan. According to cell type-dependent uptake of α-synuclein aggregates, finding more novel plasma membrane proteins regulating α-synuclein internalization is important.

Another important finding was that compared with Pre-Agg or Int-Agg, α-synuclein aggregates (Agg) were a form of α-synuclein aggregates to be efficiently internalized into neuronal cells. Accordingly, STM1D9 and MTM7A8 also potently internalized a form of Agg. Although Pre-Agg was internalized by those clones, I found that Pre-Agg was also mixture of monomeric form and partially aggregated form of α-synuclein under TEM. Thus, I believed that Pre-Agg was internalized into SH-SY5Y cells. It has been a question of great interest which forms of α-synuclein would be propagated. Recently, Jones et al. (2015) showed that both sarkosyl-insoluble and soluble fractions of brain homogenate from Lewy body disease patients cause CNS pathology in mice when inoculated. Accordingly, there is abundant room for further study to characterize this subject. Strictly

speaking, which forms of α-synuclein, dimer or low-n oligomer, and wild-type or mutant form of α-synuclein could propagate should be further addressed. Then, additional studies on the role of STM1D9 and MTM7A8 for this purpose and *in vivo* studies are needed.

It is interesting to note that the seeding effect of aggregated α-synuclein on the propagation was evidently shown in the BiFC model, as adding *in vitro* aggregates to cell culture medium increased the number of cells with the BiFC fluorescence. Prior study utilizing this BiFC system have noted similar effect as cell passages go by during continuous culture (Bae et al., 2014). On the one hand, increasing the BiFC fluorescence by treating α-synuclein aggregates as seeds was useful for better characterization of the putative clones in the propagation of α-synuclein aggregates in the BiFC system.

This study set out to isolate membrane proteins which stimulate α-synuclein internalization and more detailed studies will be performed to characterize the receptors or channels responsible of α-synuclein in future. Future studies should show the binding of these candidates to α-synuclein, signaling pathway, and mechanism of the internalization. Also, it would be interesting to assess the effect of these candidates *in vivo* by generating knockout mice and inoculating the pathogenic α-synuclein into that knock-

out mice to test its propagation.

This study elucidated an important part of the mechanism for the propagation of synucleinopathies. Researches on the propagation of α-synuclein pathology are important in the context of therapeutic aspect. At the aspect of gain of function activities, α-synuclein is related to membrane permeabilization and impairment of proteasome and ER-golgi trafficking. At a loss of function, abnormal α-synuclein may cause inhibition of vesicle docking (Dhay et al., 2015). In both cases, abnormal α-synuclein has toxic effects on the affected neurons. This is why antibody therapy targeting extracellular α-synuclein has been studied by many researchers (Bae et al., 2012; Tran et al., 2014). However, several limitations, such as problem in delivery across the BBB or risks of inflammation, could exist when using antibody therapy (Jolesz, 2014). Therefore, identification of α-synuclein receptors that mediate the internalization of α-synuclein enables to provide a new mechanism-based approach for the treatment of synucleinopathies by inhibiting the membrane proteins.

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국문초록

알파시뉴클레인(α-synuclein)의 transmission 은 최근 파킨슨병(Parkinson's disease), 루이소체치매(dementia with Lewy bodies), 다계통위축증(multiple system atrophy)을 포함하는 synucleinophathy 분야에서 활발히 연구되고 있다. 하지만 뉴런에서의 transmission 을 매개하는 막단백질에 대해서는 연구가 많이 이루어지지 않았다. 따라서 세포실험을 기반으로 한 어세이를 개발함으로써 스크리닝을 통해 알파시뉴클레인의 transmission 에 관여된 막단백질을 동정하고자 하였다. 알파시뉴클레인은 대장균을 통해 발현시킨 후 정제되었고, 이를 소니케이션과정을 포함한 총 21일동안 인큐베이션함으로써 *in vitro* 에서 응집체를 형성시켰다. 만들어진 알파시뉴클레인 응집체에서 섬유성 구조가 형성되었음을 투과전자현미경사진을 통해 확인하였다. 또한 알파시뉴클레인은 Alexa Fluor 488 형광으로 표지되었다. 표지된 알파시 뉴클레인을 이용하여 SH-SY5Y 세포로의 유입을 관찰하였는데, 클라트린 매개 엔도사이토시스(clathrin-mediated endocytosis)를 저해하는 고장성(hypertonic)의 sucrose 조건과 마크로피노사이토시스(macropinocytosis)를 저해하는5-(N-Ethyl-N-

isopropyl) amiloride 를 처리한 조건에서 그 유입이 감소하였다. 이는 특정 막단백질이 알파시뉴클레인의 세포 내 유입을 매개할 가능성을 제시한다. 또한 막단백질을 발현산물로 하는 수천개의 cDNA 를 포함한 라이브러리를 구축하였으며, SH-SY5Y 세포를 multi-well 배양접시에서 키운 후 각각의 cDNA 를 발현시킨 후, 이 세포를 알파시뉴클레인 응집체에 노출시켰다. 이와 같은 스크리닝 방법을 통하여, 알파시뉴클레인의 세포 내 유입을 촉진하는 아홉개의 후보 유전자를 분리해냈다. 그 중에서도 STM1D9와 MTM7A8이 효과적이었다. 한편으로, 이들 유전자의 과발현 실험 결과, 타우(tau) 응집체와 베타아밀로이드(amyloid beta) 올리고머(oligomer)는 세포 내 유입을 증가시킨다고 할 수는 없었다. STM1D9와 MTM7A8의 발현은 SH-SY5Y/BiFC system 에서 Venus1-α-synuclein 과 α-synuclein-Venus2 의 세포간 transmission 또한 유의미하게 증가시켰다. 반대로 세포 내 STM1D9와 MTM7A8의 발현을 억제했을 때에는 알파시뉴클레인 응집체의 세포 내 유입이 감소하였다. 이러한 결과는 STM1D9와 MTM7A8가 신경세포에서의 transmission 과정 중 알파시뉴클레인 응집체의 세포 내 유입에 역할을 하는 것을 시사한다.