이학석사 학위논문

알츠하이머병에서 뉴런 간 Tau 확산 조절 인자에 대한 연구

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이 논문을 이학석사 학위논문으로 제출함

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ABSTRACT

Tau Pathology Modulator Promotes Tau Propagation and Uptake in Neurons in Alzheimer's Disease

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Alzheimer's Disease (AD) is characterized by Amyloid beta (Aβ) plaques and tau tangles that cause neuronal death and dementia. Tau propagation has garnered attention in AD research for its strong correlation with disease progression. Severity of neurodegeneration in AD is in line with the transneuronal spread and deposition of pathological tau, which occurs stereotypically from the entorhinal cortex to the entire cerebral cortex via the hippocampus. Tau propagation is a commonly observed phenomenon not only in AD but

also in various tauopathies. Despite extensive research on tau propagation over the recent years, the mechanism underlying tau propagation remains enigmatic.

The purpose of identifying tau propagation modulators was to understand tau propagation and discover a potential therapeutic target in AD. Inhibiting interneuronal tau propagation can fundamentally transform the approach of AD therapy that is currently more focused on Aβ-targeted therapy. I used the tau-BiFC propagation cell-based assay to screen 1,806 genes encoding secretory proteins. Here, I show that, among several genes that have demonstrated their effect on tau propagation, TauSec protein increases internalization and propagation of tau in SH-SY5Y and mouse primary cortical neurons. TauSec promotes tau internalization in neurons via interaction with its receptor, which has been reported to be involved in the assembly of V-type ATPase. Treatment of TauSec protein leads to accumulation and modification of the TauSec receptor in neuronal cells. On the other hand, knockdown of TauSec receptor in neuronal cells significantly reduces tau uptake in neuronal cells, even in the presence of TauSec. Prolonged TauSec incubation leads to increase in lysosomal pH and accumulation of intracellular tau protein, indicating dysregulation in lysosomal acidification and leading to defective tau clearance in neurons. In addition, blocking TauSec receptor with anti-TauSec receptor

antibody or small molecule inhibitor (RRPPI) impairing the TauSec-TauSec receptor interaction abolishes tau uptake and tau propagation in neuronal cells. Together, the results of this research identify TauSec as a potent modulator of tau propagation, hence its value as a therapeutic target for AD and tauopathies.

Keywords : Alzheimer's Disease, Tauopathy, Tau propagation, Tau internalization, Bimolecular Fluorescence Complementation
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ABBREVIATIONS

Aβ	Amyloid beta
AD	Alzheimer's Disease
ATP6AP2	ATPase H+ Transporting Accessory Protein 2
Baf.A1	Bafilomycin A1
BiFC	Bimolecular Fluorescence Complementation
DMSO	Dimethyl sulfoxide
EIPA	5-(N-Ethyl-N-isopropyl) amiloride
ERK	Extracellular-signal-regulated kinase
H_2O_2	Hydrogen peroxide
HMW-Tau	High molecular weight Tau oligomers
LiCl	Lithium Chloride
LMW-Tau	Low molecular weight Tau oligomers
MβCD	Methyl-β-cyclodextrin
MB	Methylene blue
NFT	Neurofibrillary tangle
PBS	Phosphate-buffered saline
PMSF	Phenylmethyl sulfonyl fluoride
PVDF	Polyvinylidene fluoride
RAGE	Receptor for advanced glycation end products
RAS	Renin-angiotensin (pathway)
s.e.m.	Standard error of mean
SDC1	Syndecan 1
SDS-PAGE	SDS-Poly Acrylamide Gel Electrophoresis

VC	Venus C-terminal
VN	Venus N-terminal
V-ATPase	Vacuolar-type H+ -ATPase
WT	Wild type

INTRODUCTION

Alzheimer's Disease and tau pathology

Alzheimer's Disease (AD) is the leading cause of dementia and is characterized by tau neurofibrillary tangles (NFTs) and Amyloid beta (Aβ) plaques (Knopman et al., 2021). While early efforts for AD therapy were focused on eliminating Aβ plaques, such therapeutics showed little clinical success (Sevigny et al., 2016). Therefore, recent efforts have been steering toward targeting tau aggregates, which spread and deposit throughout the brain in correlation with cognitive decline (Bell et al., 2020). Microtubule Associated Protein Tau (MAPT) is an intrinsically disordered protein whose physiological role is to stabilize microtubules (Zhu et al., 2015). In pathological situations, tau proteins are misfolded and aggregate into NFTs, which, in many cases, is preceded by hyperphosphorylation of tau. Phosphorylation in physiological settings can contribute to its affinity to microtubules, yet in pathological settings, tau becomes hyperphosphorylated, which causes it to be detached and form aggregates (Spillantini et al., 2013).

Tau propagation

Tau propagation is observed in AD brains, where pathological,

aggregated tau spreads from neuron to neuron in a prion-like manner, inducing misfolding of tau in recipient cells. (Takeda, 2019) In the early stages of AD, tau aggregates are observed in the entorhinal cortex. As the disease progresses, tau aggregates spread throughout the hippocampus to the cortex in a stereotypical pattern (Braak et al., 2006).

Although tau propagation has been researched extensively, the mechanism remains yet to be elucidated. There are, however, numerous hypotheses that attempt to explain parts of the process. Misfolded tau can be secreted from neurons via exosomes, ectosomes or as free tau crossing the plasma membrane by translocation (Merezhko et al., 2020). According to Tardivel et al., tau can also cross between neurons via nanotubes (Tardivel et al., 2016). Detection of tau in ISF and CSF in mouse models and humans with tauopathies is an indication that tau is, indeed, released from cells (Samans et al., 2012). Secreted tau that exists outside of cells is internalized by recipient cells by macropinocytosis or clathrin-dependent endocytosis, or membrane fusion of exosomal vesicles. Tau was reported to be co-local with endosomal markers, and endocytosis inhibitors have shown to decrease tau internalization in neurons (Wu et al., 2013). As tau is transmitted from one neuron to another as so, pathological tau serves as seeds that induce misfolding and aggregation of normal tau in recipient neurons

(Holmes et al., 2014).

A handful of genes have been identified to affect tau propagation. Heparan sulfate proteoglycans (HSPG) promote macropinocytosis of tau and increase the uptake of not only tau but also alpha-synuclein in Parkinson's Disease (Holmes et al., 2013). Bin1, which is an AD risk gene, has been discovered to indirectly prevent tau propagation by inhibiting clathrin-mediated endocytosis (Calafate et al., 2013). LRP1, which was recently discovered as a specific regulator of tau propagation, specifically increases uptake of tau proteins (Rauch et al., 2020). Also, RAGE (receptor for advanced glycation end products) was also recently discovered as a receptor for tau internalization and propagation (Kim et al., 2022). Although the discovery of these genes enhanced our understanding of tau propagation, they fail to fully explain the overall process.

Majority of tau-targeted AD drugs that are undergoing clinical trials are focused on post-translational modifications, aggregation, and clearance of tau. The potential that anti-tau propagation medication may have on curing and delaying AD progression calls for additional research on this field. Furthermore, prion-like protein propagation is observed in other diseases such as Parkinson's Disease and Huntington Disease. Therefore, this study may have significance beyond the scope of AD and tauopathies.

Limited methods to study Tau propagation

The reason that tau propagation remains a mystery today is mainly the lack of appropriate assays that accurately demonstrate tau propagation *in vitro* and *in vivo*. Co-culturing neurons and staining to observe tau transmission is not only complicated and time-consuming, but it makes it impossible to observe tau propagation over time in live cells (Calafate et al., 2016). Also, Furman et al., demonstrates a FRET-based biosensor assay to observe tau in a cell-based system (Furman et al., 2017). However, this system requires treating high concentrations of external tau in order to observe tau FRET puncta in cells and is, therefore, a very artificial system. Also, large-scale screening experiments are unrealistic for this assay.

In vivo methods to study tau propagation are even more limited. Currently, there is a mouse model that overexpresses tau only in the entorhinal cortex, in which after 12~21 months, tau propagation is observed in the hippocampus and cortex. This requires an extremely long period of time, delaying research progress, and it is an artificial condition because it is an overexpression model (deCalignon et al., 2012). Other methods include obtaining tau seeds from human patients and injecting them to observe tau propagation immunohistochemically (Audrain et al., 2018) and viral transduction of fluorescent tag-fused tau into mice

(Clavaguerea et al., 2013).

Risk factors of dementia – Cardiovascular diseases

There are various causes to dementia, including AD, Vascular dementia (VD), and Frontotemporal dementia (FTD). AD is the leading cause of dementia, summing up to about 60~70%, followed by VD, which is found in 20~30% of dementia cases (Holtzman et al., 2011). What complicates dementia is that these causes are not always independent. Instead, they are often intertwined. In the clinic, AD is frequently accompanied by cardiovascular lesions, and AD-related pathologies are often found in VD patients, making the two difficult to distinguish at times. VD occurs acutely, usually caused by stroke or other cardiovascular diseases, and the progression occurs step-wise (Bir et al., 2021). The direct cause of AD is unknown, as it occurs and develops gradually, over decades. Numerous risk factors for AD have been found, including but not limited to hypertension, diabetes, stress, and alcohol consumption.

The correlation of hypertension and dementia have been repeatedly reported through clinical studies, but most of them are focused on vascular dementia. However, numerous observational studies report the correlation between hypertension and specifically, AD. Yet, the mechanism is not as clear as it is for VD. In hypertensive patients, the risk for AD increases 1.5 times (Ou et al.,

2020). Also, post-mortem brains of hypertensive patients demonstrated increase in amyloid beta plaques and tau tangles. Also, the renin-angiotensin (RAS) system, which regulates blood pressure, is overactivated in dementia patients (Kivipelto et al., 2002). Interestingly, patients who took anti-hypertensive medication that tackle the RAS pathway for extended periods of time have a 35~40% lower chance of developing dementia (Wolozin et al., 2008). These clinical evidences suggest that hypertension and the RAS pathway can provide a new and effective approach to AD therapy.

RAS in dementia

RAS is known to regulate blood pressure and homeostasis, starting with renin, which is produced in kidney's juxtaglomerular cells, producing angiotensin that induces the secretion of aldosterone (Ribeiro, de Souza, and Silva, 2020). Recently, alternative axes of RAS have been discovered. RAS components cannot cross the BBB (blood-brain barrier), therefore de novo local synthesis of RAS components has been proposed and reported (Forrester et al., 2018). Brain RAS has been reported to be involved in not only local blood pressure regulation but also AD pathology (Loera-Valencia et al., 2021).

Renin is the rate-limiting step of RAS, as it is an aspartyl protease that cleaves angiotensinogen to generate angiotensin I. Its

activity is regulated by two ways. First is mechanically cleaving the pro-segment, which is about 43 amino acids long. The pro-form of renin has very low enzymatic activity because the pro-segment functions as a flap that covers the active site of renin, preventing its substrate, angiotensinogen, from binding (Nabi et al., 2009). The second way is binding to the renin receptor. The pro-segment contains a binding site to renin receptor; therefore, when binding occurs, the active site of renin is exposed (Nguyen and Muller, 2010).

In the brain, renin has been identified on mostly neurons and some in astrocytes (Lee-Kirsch et al., 1999; Hirose et al., 1980; Lavoie et al., 2004; Jackson et al., 2018), although it is still controversial with some claiming that the detected renin molecules are remnants of blood trapped in brain tissue (van Thiel et al., 2017). Renin is generally upregulated in hypertensive patients (Saxena et al, 2018), but the regulation of brain renin expression remains enigmatic today.

The renin receptor, which is also known as ATP6AP2 (ATPase H+ Transporting Accessory Protein 2), is a V-type ATPase accessory protein that is essential in the assembly of V-type ATPase complex. Knockdown of renin receptor suppresses the expression of V_0 subunits of V-ATPase, resulting in deacidification of lysosomes (Kinouchi et al., 2010), and leads to embryonic lethality in mice (Sihn et al., 2010). Also, renin receptor activates intracellular pathways,

such as extracellular regulated protein kinase (ERK) pathway or Wnt signaling (Nguyen et al., 2002; Cruciat et al., 2010). Renin receptor is abundantly expressed across various cell types (Ichihara and Yatabe, 2019), and it is also widely expressed in the brain, in neurons (Schafer et al., 2013), astrocytes (Garrido-Gil et al., 2013), and microglia (Valenzuela et al., 2010). In hypertensive patients, renin receptor expression in the brain is increased (Mohsin et al., 2020). Also, according to GEPIA 2 (Gene Expression Profiling Interaction Analysis), renin receptor expression has a strong positive correlation with tau. These findings call for further research on renin and renin receptor in the brain to uncover their interaction and role in AD.

To uncover the mechanism underlying tau propagation and identify tau propagation modulators, I characterized a cell-based tau BiFC propagation assay and performed genome-wide functional screening of secretory protein library. Here, I show that some RAS components may play a crucial role in tau propagation in neuronal cells.

MATERIALS AND METHODS

Cell culture and DNA transfection

Mouse primary cortical neurons were prepared from embryonic day 16-20 of C57bLL6/J mice as described by Kam et al., 2013. Cells were dissociated by trypsin-EDTA and grown in Neurobasal Medium (Invitrogen) supplemented with 2% B27 supplement (Invitrogen) and 1% GlutaMAX^{TM}-I (Invitrogen), along with 100 μg/mL Penicillin & Streptomycin (Invitrogen), and 10 μg/mL Gentamycin (Gibco®). Partial medium changes were performed every 3 days, and cells were grown for 7 days *in vitro* (DIV) before experiments. SH-SY5Y cells were cultured in Dulbecco's modified Eagle medium (Hyclone) supplemented with 10% fetal bovine serum (Gibco®), 100 μg/mL Penicillin/Streptomycin (Invitrogen), 10 μg/mL Gentamycin (Gibco®). Transfection was done using Lipofectamine 2000 reagent (Invitrogen) for primary neurons and Lipofector-pMAX transfection reagent (AptaBio) for SH-SY5Y cells, following the manufacturere's instructions.

Chemicals and reagents

DQ-red BSA (D12051) (Invitrogen); DyLight 488 NHS Ester (Thermo Scientific); His-renin (ab135012) (Abcam); L4000 bioactive compound library (TargetMol); Bioactive compound library

(Targetmol Inc.); 48562 (Z46190510) (Enamine); were purchased.

Antibodies

The following antibodies were used for western blotting, immunoprecipitation, and immunocytochemistry: GST (sc-53909), His (sc-803), ANTI-FLAG M2 Affinity Gel (A2220), and Renin(sc-133145) (Santa Cruz); ATP6AP2 (AF5716) (R&D Systems); FLAG (F1804) (Sigma Aldrich); Renin (A1585), ATP6AP2 (A6531) (ABClonal); t-ERK, p-ERK, Actin Beta;

TauSec receptor knockdown

To knock-down TauSec receptor in SH-SY5Y cells, lentiCRISPR v2 vector was used. Plasmids were transfected into wild-type SH-SY5Y cells. After 24h, cells were selected with 2 μg/mL puromycin for 48-72h. Cell monoclones were acquired by limiting dilution, and knockdown was confirmed by western blot. Validated cell lines were used for further experiments.

Knock-down of the TauSec receptor in primary cortical neurons were conducted by shRNA. Lenti-HEK cells were used to generate shRNA viruses, which were introduced to primary cortical neurons at DIV 7. Receptor knockdown was confirmed by western blot.

Immunocytochemistry (ICC) and fluorescent microscopy

Cells were fixed with 4% paraformaldehyde for 15 min at room temperature. After washing three times with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS, and blocked with 5% fetal bovine serum in PBS for 1 h in room temperature. Then, cells were incubated with primary antibodies in PBS overnight in 4°C. Fluorescent-conjugated secondary antibodies in PBS for 2 h in room temperature. DQ-Red BSA (Invitrogen) was added to examine functional lysosomes, or DAPI was added to stain nuclei. Images were captured on a Zeiss LSM700 laser scanning confocal microscope or a fluorescence microscope (Olympus).

Expression and purification of GST- or His-tag fusion proteins

For purification of GST- or His-tag fusion proteins, protein-coding constructs were each expressed in BL21 cells and inoculated with IPTG at 37°C for 3 h. Cells were resuspended in GST binding buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1 mM EDTA, and 1 mM PSF), or His-binding buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM imidazole, and 1 mM PSF). Resuspended cells were lysed by sonication. Tau was boiled at 100°C for 20 min. Then, lysates were centrifuged for 13,000 rpm for 25 min. The supernatant was filtered through a 0.2 μm filter and loaded onto a column with Ni-NTA agarose beads for His-affinity, and Glutathione beads for GST-affinity chromatography. The column was washed 7 times, and the

bound protein was eluted using 5 mM glutathione or 300 μM imidazole. Purified protein was stored at -20°C until use.

For fluorescence conjugation, 1 mg of His-tau was incubated with a single vial of Dylight-488 or Alexa-594 for 1.5 h in room temperature. Then, protein was dialyzed in a Snakeskin Dialysis Tubing (Thermofisher) membrane submerged in PBS overnight.

In vitro production of Tau oligomers

His-0N4R tau proteins were purified as mentioned above. Tau monomers, Heparin, and DTT were mixed together at final concentrations of 48 μM, 12 μM, and 5 mM, respectively in PBS. Then, the mixture was incubated at room temperature for 1 h. After 1 h, the mixture consists of mostly low-molecular weight oligomers. Half of the mixture was stored at -20°C, and the remaining half was further incubated at room temperature for 20 min to form high-molecular weight oligomers. Low- and high-molecular weight Tau oligomers were mixed to 1:1 ratio before use.

For fibril assembly, Tau solution was made with the same composition and was incubated at 37°C, 1,000 rpm for 24-48 h.

Tau-BiFC propagation assay

For primary screening, SH-SY5Y/ VN-tau cells and SH-SY5Y/tau-VC cells were co-cultured. After 24 h, they were transfected with

RFP-N1 and each of 1,806 cDNAs encoding secretory proteins. After 48 h, cells were observed under a fluorescence microscope, and images were obtained for analyses. The BiFC intensity measured by ImageJ (NIH) was an indication how much Tau propagation had occurred.

Western blot

Cell samples were lysed by sonication in ice-cold RIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1mM EDTA, 1.1% NP-40, and 0.25% sodium deoxycholate) with 1mM phenylmethylsulfonyl fluoride (PMSF) added right before use. After centrifugation, supernatant was subjected to SDS-PAGE (polyacrylamide gel electrophoresis) and transferred to polyvinylidene fluoride (PVDF) membrane by using Semi-Dry transfer system WSE-4025 HorizeBLOT 2M-R (ATTO, Tokyo, Japan). Blots were blocked by 3% (w/v) bovine serum albumin (BSA) solution in TBS-T and subjected to western blot. Immunoreaction was detected via chemiluminescence method.

Virus production

At 70~80% confluency, Lenti-HEK293 cells were transfected with DNA construct of interest along with PAX2 and pVSVg constructs. After 12 h, media were replaced with fresh media, and after 48 h, media with viral particles were collected. Collected media were

filtered through a 0.45 μM filter to remove any cells or impurities. 3 volumes of Lenti-X^{TM} Concentrator (Takarabio) was added to 1 volume of collected media. Then, I followed the vendor's manual to retrieve viral particles from the media.

ELISA

GST-TauSec (10 nM) was coated onto microwell plates in 0.1M carbonate buffer (pH 9.6) over-night at 4°C. Wells were washed with PBS 5 times and blocked with 3% BSA in PBS for 30 minutes at room temperature. Simultaneously, His-TauSec receptor (1 μM) was mixed with varying doses of RRPPI in PBS and incubated at room temperature. After blocking, wells were washed, and mixtures of proteins with or without RRPPI were added to each well. After 1hr incubation at room temperature, wells were washed 3 times. Then anti-His antibody was added (1:500) and incubated at room temperature for 2 h. Wells were washed 3 times, and biotinylated rabbit antibody (1:2000) was added and incubated at room temperature for 1 h. Wells were washed 5 times, and streptavidin solution (100x) diluted in diluent was added. After 30 minute-incubation, cells were washed 3 times. TMB solution was added and incubated for 15 min, after which equal volumes of stop solution were added to stop the reaction. Binding strength was measured by the microwell plate reader that read microwell plates' values at 450

nM.

Quantification and statistical analysis

All values in graphs are demonstrated as mean ± standard error of the mean (s.e.m.). Statistical analysis was performed by GraphPad Prism software (Version 9.0.1, GraphPad, La Jolla, CA). Statistical significance was determined by two-tailed t test for groups of two and two-way ANOVA for groups of three or more. Significant levels are as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

RESULTS

Characterization of Tau-BiFC propagation assay to monitor Tau propagation

Tau-BiFC propagation assay was designed to establish a cell-based system where tau propagation can be observed and modulated (Fig. 1A). Two individual cell lines were created in SH-SY5Y human neuroblastoma cells: SH-SY5Y/VN-tau cell line which stably overexpresses the N-terminal half of Venus fused to tau (VN-tau) and SH-SY5Y/tau-VC cell line which overexpresses tau fused to the C-terminal half of Venus (Tau-VC) (Fig. 1B). When cultured individually, these cells do not generate fluorescence because half fragment of Venus is expressed. It is known that tau could form oligomers and fibers in cells after hyper-phosphorylation under diverse conditions.

When cultured together, however, fluorescence emission can be observed, if tau propagates from one cell to other cell and form multimers in the same cells. As expected, binary fluorescence complementary (BiFC) fluorescence was observed in some cells when SH-SY5Y/VN-tau cells and SH-SY5Y/tau-VC cells were cultured together (Fig. 1C). Staining cells with human tau antibody (HT7) validated that the BiFC fluorescence co-localized with tau (Fig. 1C). In order to characterize this assay, I transfected SDC1, a family

of heparan sulfate proteoglycans that are known to increase tau internalization (Holmes et al., 2013), and RAGE, a tau receptor (Kim et al., 2022), as positive controls. Overexpression of these genes led to an increase in fluorescence intensity. These results verify tau-BiFC propagation assay as a plausible representation of tau propagation in pathological circumstances.

Screening secretory protein pool to identify potential tau propagation modulator genes

This tau-BiFC propagation assay was used to identify new genes that may modulate tau propagation. The Human Secretion Protein Library from GeneCopoeia^{TM} was subjected to screening. It contains a wide array of secretory proteins, including chemokines, cytokines, hormones, enzymes, and more. Approximately 1,806 genes were screened via this assay by overexpressing individual genes and comparing their fluorescence intensity with that of control (RFP-N1 empty vector). Equal numbers of SH-SY5Y/VN-tau cells and SH-SY5Y/tau-VC cells were plated in 96-well plates and then co-transfected with RFP-N1 empty vector and each gene encoding a secretory protein. RFP-N1 served as transfection control. After 48 h following the transfection, fluorescence intensity per RFP-positive cell was measured by ImageJ. Overexpressed RAGE and SDC1, positive controls, enhanced tau-mediated fluorescence in the BiFC

assay (Fig. 2A).

From primary screening, 172 genes were selected from 1,806 cDNAs. From subsequent secondary screening of 172 genes, 38 putative positive clones were isolated (Fig. 2B). In the primary and secondary screening, cDNA clones were selected as positive clones if they repeatedly demonstrated significant positive or negative changes in the tau-BiFC fluorescence intensity (Fig. 2C). From the secondary screening results, TauSec was chosen because it repeatedly caused the largest increase in tau-BiFC fluorescence (Fig. 2D).

Purified TauSec increases tau propagation in neuronal cells

TauSec is known as a secretory protein, GST-TauSec protein was expressed in E. coli and purified. Treatment of cells with tau-BiFC propagation assay with TauSec enhanced the fluorescence in tau-BiFC propagation assay (Fig. 3A, 3B). Tau propagation was also verified by another assay utilizing pLenti-DsRed_IRES_MAPT:EGFP in mouse primary cortical neurons. In this tau-lentivirus assay, tau was fused to EGFP, and RFP is expressed in the same construct. Primary cortical neurons infected with this virus display both red and green fluorescence. However, when tau-EGFP propagates to a neighboring non-infected neuron, the recipient cell emits GFP fluorescence only (Fig. 3C). When primary cortical neurons were

treated with nanomolar doses of GST-TauSec protein, the number of GFP-only neurons increased, indicating that TauSec treatment enhances tau propagation in neurons (Fig. 3D, 3E).

TauSec increases tau uptake in neurons

To identify the mode of propagation modulation, tau uptake assay was performed. SH-SY5Y cells were treated with purified human oligomeric 0N4R tau proteins labeled with fluorescent Dylight 488 (tau-Dylight488, 250 nM) for 2 h in the presence of GST-TauSec (1 nM). Remarkably, GST-TauSec increased tau internalization in SH-SY5Y cells (Fig. 4A, 4B). Similar experiments were performed in primary cultured neurons. Treating mouse primary cortical neurons with GST-TauSec increased internalization of tau-Dylight 488 oligomers (Fig. 4C, 4D). Uptake of purified tau protein by SH-SY5Y cells that do not express endogenous tau was also observed by western blot (Fig. 4E).

I then assessed the pathways of tau internalization. The mechanism of tau uptake by TauSec was investigated using endocytosis inhibitors, EIPA and Dynasore, which inhibit macropinocytosis and clathrin-mediated endocytosis, respectively. When clathrin-mediated endocytosis was blocked by Dynasore, TauSec was not able to promote tau internalization (Fig. 4F), indicating that tau is internalized into neurons through the clathrin-

mediated endocytosis. In addition, decrease in tau-BiFC fluorescence in the presence of such endocytosis inhibitors suggest that enhancing tau uptake in neurons promotes tau propagation between neurons.

TauSec expression in neurons is increased in response to various stress conditions.

TauSec is known to play a key role in RAS pathway and expressed mostly in the kidney (Paul, Wagner, and Dzau, 1993). However, growing evidence indicates that brain RAS does exist (Phillips and de Oliveira, 2008). The physiological function and characteristics of brain TauSec remain enigmatic due to its low expression. However, expression of TauSec in the brain has been confirmed across various species without an understanding of its role. As reported, I was able to detect TauSec expression in neurons by western blot and qRT-PCR analysis (Fig. 5A). Interestingly, I could observe that TauSec expression is regulated under various stress conditions, including salts (Fig. 5A).

Sodium levels in cerebrospinal fluid (CSF) are higher in hypertensive patients with a family history of AD (Souza et al., 2020). To mimic high salt condition, primary cortical neurons were treated with 1 mM NaCl. As an *in vitro* model of AD pathology, neurons were exposed to amyloid beta oligomers (Aβ, 5 μM) and tau oligomers (oTau, 500 nM). As oxidative stress, neurons were exposed to H_2O_2

(100 μM). Interestingly, I found that TauSec expression was increased by the treatment with NaCl, Aβ, oTau and H_2O_2 in primary cortical neurons at protein level (Fig. 5A, 5B) and RNA level (Fig. 5C). While it was difficult to observe endogenous TauSec by immunostaining mouse in primary cortical neurons, TauSec receptor was detected and was increased when TauSec was overexpressed in neurons (Fig. 5D). Unlike primary neurons, TauSec levels were not altered by the above stress conditions in SH-SY5Y cells (data not shown).

TauSec increases the amount and modification of the TauSec receptor

TauSec binds to TauSec receptor, which is also known as a V-type ATPase accessory protein. When SH-SY5Y cells were treated with purified TauSec, TauSec receptor protein accumulated in the cell, and appeared in a slowly migrating form (Fig. 6A). This shift in migration happened as early as 4 h of TauSec treatment, and level of TauSec receptor increased with time, indicating that TauSec increases TauSec receptor level and modification at protein level. I confirmed that TauSec activates its downstream signals, including the ERK pathway (Fig. 6B).

I then analyzed the nature of TauSec receptor modification using phosphatase and glycosidase. Treatment of cell extracts with phosphatase did not affect the shift of TauSec receptor (data not

shown). In contrast, incubation of cell extracts with PNGase F, a N-glycosidase enzyme, reduces the up-shifted band of TauSec receptor (Fig. 6C). These results indicate that slowly migrating protein of TauSec receptor is a N-glycosylated form of TauSec receptor. In addition, qRT-PCR analysis revealed that TauSec did not increase level of TauSec receptor mRNA (Fig. 6D), indicating that the accumulation of TauSec receptor by TauSec results from regulation of post-transcriptional level.

TauSec receptor is essential in TauSec-mediated tau uptake

To examine the role of TauSec receptor in tau uptake in neuronal cells, expression of TauSec receptor was reduced by CRISPR-Cas9 system in SH-SY5Y cells. From western blot analysis, I could isolate 3 different TauSec receptor knockdown cells (sgTauSecR#1, 2 and 3) (Fig. 7A). Subsequently, I found that tau internalization was significantly reduced in all three TauSec receptor knockdown cell lines compared to that of control cells (Fig. 7B, 7C).

In addition, I examined the role of TauSec receptor in tau uptake. I generated lentivirus harboring two TauSec receptor shRNA#1 and 2 (lenti-shTauSecR #1 and 2). Then, primary cortical neurons were infected with such lentiviruses and analyzed by western blotting. I found that TauSec receptor was reduced by lenti-shTauSecR #1 and 2 (Fig. 7D). In tau-uptake assay, I found that the

internalization of tau-pHRhodo oligomers was abolished in TauSec receptor-deficient primary neurons (Fig. 7E, 7F). Even in the presence of TauSec, tau uptake was not increased (Fig. 7E, 7F). All of these results suggest that TauSec receptor plays an essential role in tau uptake in neuronal cells.

TauSec-receptor binding is more crucial than angiotensin production

TauSec plays two roles, as a ligand binding to TauSec receptor, and as an aspartyl protease that cleaves angiotensinogen to generate angiotensin I. I decided to study whether TauSec receptor signaling or angiotensin-receptor signaling is important for tau uptake in neuronal cells. Thus, I generated a TauSec mutant without its pro-segment (active TauSec), which exhibits increased protease activity but lower binding affinity to TauSec receptor (Fig. 8A).

I purified GST-WT proTauSec and GST-active TauSec proteins from *E. coli*. (Fig. 8B). While treatment with WT TauSec protein enhanced tau uptake in SH-SY5Y cells, active TauSec could not increase tau uptake as much as WT proTauSec (Fig. 8C, 8D), losing the stimulatory effect of TauSec in tau uptake into neuronal cells. Because the lack of pro-segment reduces TauSec's affinity to TauSec receptor, I could conclude that the binding of TauSec to TauSec receptor is important in inducing tau internalization.

Prolonged exposure to TauSec leads to tau accumulation in neurons

I then addressed how TauSec-receptor interaction affects tau uptake and propagation in neuronal cells. Since tau uptake usually occurs in a relatively short time within a couple of hours, I examined the long-term effect TauSec may have on tau pathology. When primary neurons were treated with 10 nM GST-TauSec for 48 h, I found that tau protein is significantly accumulated as examined with western blot analysis (Fig. 9A. 9B). In addition, this accumulation of tau was also observed in SH-SY5Y cells overexpressing tau-VC after exposure to TauSec for 48h and 72 h. Thus, I suggest that tau accumulation is not a result of increased tau expression but rather accumulation of intracellular tau. The amount of accumulated tau in TauSec-treated cells was comparable to that of bafilomycin, lysosomal V-ATPase inhibitor, or MG132, proteasome inhibitor-treated cells (Fig. 9C).

TauSec impairs lysosomal pH and activity in neuronal cells.

Because TauSec receptor is involved in the assembly of V-type ATPase, I hypothesized that prolonged exposure to TauSec could interfere with the assembly of V-type ATPase and lysosomal pH. Thus, I measured lysosomal pH using pHLare. Compared to control cells, deletion of TauSec receptor in SH-SY5Y cells increased lysosomal pH (Fig. 10A, 10B). In addition, prolonged incubation of

SH-SY5Y cells with GST-TauSec increased lysosomal pH too, albeit to a lesser degree (Fig. 10A, 10B). In addition, I used another lysosomal activity assay using DQ-Red BSA to assess lysosomal activity. As seen in the pHLare assay, Incubation of SH-SY5Y cells with TauSec for more than 48 h significantly decreased the lysosomal activity (Fig. 10C, 10D). Thus, TauSec treatment induces higher and dysregulated pH, leading to the impairment of lysosomal activity in neuronal cells.

Anti-TauSec receptor antibody decreases tau uptake in SH-SY5Y

Because TauSec-binding to TauSec receptor seems to be essential for tau uptake, I tried to inhibit such interaction by using anti-TauSec receptor antibody. Treating SH-SY5Y cells with 5 and 50 μg/mL anti-TauSec receptor antibody prior to TauSec treatment reduced TauSec-mediated ERK signaling (Fig. 11A), indicating that the antibody might block the ligand-receptor interaction. In addition, pre-treatment of SH-SY5Y cells with anti-TauSec receptor antibody greatly reduced tau internalization (Fig. 11B, 11C). This goes to confirm that TauSec should interact with the TauSec receptor to stimulate tau internalization.

RRPPI small molecule successfully blocks the TauSec-TauSec receptor binding and tau propagation

Recently, RRPPI was discovered by Loganathan et. al., (ref) in algorithm prediction assay of TauSec and TauSec receptor interaction. Their study identified RRPPI as a potential interaction inhibitor between TauSec receptor and TauSec. Thus, I decided the effect of RRPPI on tau propagation. First, I examined its effect by in vitro ELISA. GST-TauSec and His-TauSec receptor ectodomain (a.a. 17 ~ 302) proteins were expressed in *E. coli* and then purified (Fig. 12A). His-TauSec receptor ectodomain is smaller in size compared to the full-length (FL) protein expressed endogenously in SH-SY5Y (Fig. 12B). In the ELISA analysis, I found that RRPPI dose-dependently decreased the binding between GST-TauSec and His-TauSec receptor ectodomain in vitro (Fig. 12C). It seems to interfere the interaction at sub-micromolar concentration.

To confirm this effect in neuronal cells, I performed live-cell ELISA in which GST-TauSec binding to cell surface TauSec receptor was monitored. Treatment of SH-SY5Y cells with 10 μM RRPPI significantly reduced the binding of GSTY-TauSec to cell surface (Fig. 12D). In addition, RRPPI was able to reduce TauSec-induced ERK activation (Fig. 12E). These results indicate that RRPPI is able to block TauSec binding to TauSec receptor.

After characterization of RRPPI as a potential inhibitor between TauSec and TauSec receptor, I tested effect of RPPI on tau propagation using tau-BiFC propagation assay. I found that RRPPI

could diminish TauSec-mediated increase of tau propagation in SH-SY5Y cells (Fig. 13A). In addition, RRPPI was able to reduce TauSec-mediated tau accumulation to control levels in tau-VC-expressing SH-SY5Y cells (Fig. 13B, 13C). These observations suggest that RRPPI could be a hit as a promising inhibitor of TauSec-TauSec receptor interaction to ameliorate tauopathies and AD.

Figure 1. Establishment and characterization of cell-based tau-BiFC propagation assay

(A) Schematic image of tau-BiFC propagation assay. Two individual cell lines: SH-SY5Y/VN-tau and SH-SY5Y/tau-VC were generated, each overexpressing tau that is fused with N-terminal (VN) or C-terminal half (VC) of the VENUS (V) protein. Individually, these two cell-lines do not emit fluorescent signal. However, when co-cultured, tau propagation and aggregation can occur and two halves of VENUS protein come within proximity. Then VENUS reforms in its native structure and emits fluorescence.

(B) Western blot of SH-SY5Y/VN-tau cells, SH-SY5Y/tau-VC cells, and two cell lines co-cultured.

(C) BiFC fluorescence in co-cultured SH-SY5Y/VN-tau cells and SH-SY5Y/tau-VC cells. Cells were immunostained with HT7 antibody (Red) and observed under fluorescence microscope. VENUS protein emits fluorescent signal. (Green, tau-BiFC).

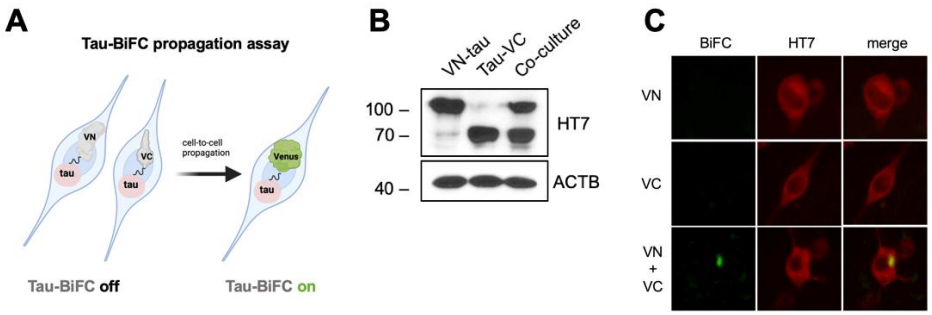

A
Tau-BiFC propagation assay
VN
tau
VC
tau
cell-to-cell
propagation
Venus
tau
Tau-BiFC off
Tau-BiFC on
B
VN-tau
Tau-VC
Co-culture
100 –
70 –
40 –
HT7
ACTB
C
BiFC
HT7
merge
VN
VC
VN
+
VC

Figure 2. Genome-wide functional screening of human secretome identified tau propagation modulators

(A) Timeline of the screening process. SH-SY5Y/VN-tau cells, SH-SY5Y/tau-VC cells were co-cultured for 24 h after cell plating and transfected for 48 h with each cDNA expressing secretory proteins. RAGE and SDC1 were used as positive controls. BiFC fluorescence was then observed under a fluorescence microscope, and fluorescence intensity was measured by ImageJ.

(B) Screening results. A total of 1,806 genes (GeneCopoeia^{TM}) were screened, and 172 putative positive clones were selected from primary screening, and 38 of them were isolated from secondary screening.

(C) Tau-BiFC images of the clones selected by secondary screening.

(D) Screening results on a graph. TauSec was identified as a potential tau propagation modulator that greatly increased tau propagation in the tau-BiFC propagation assay.

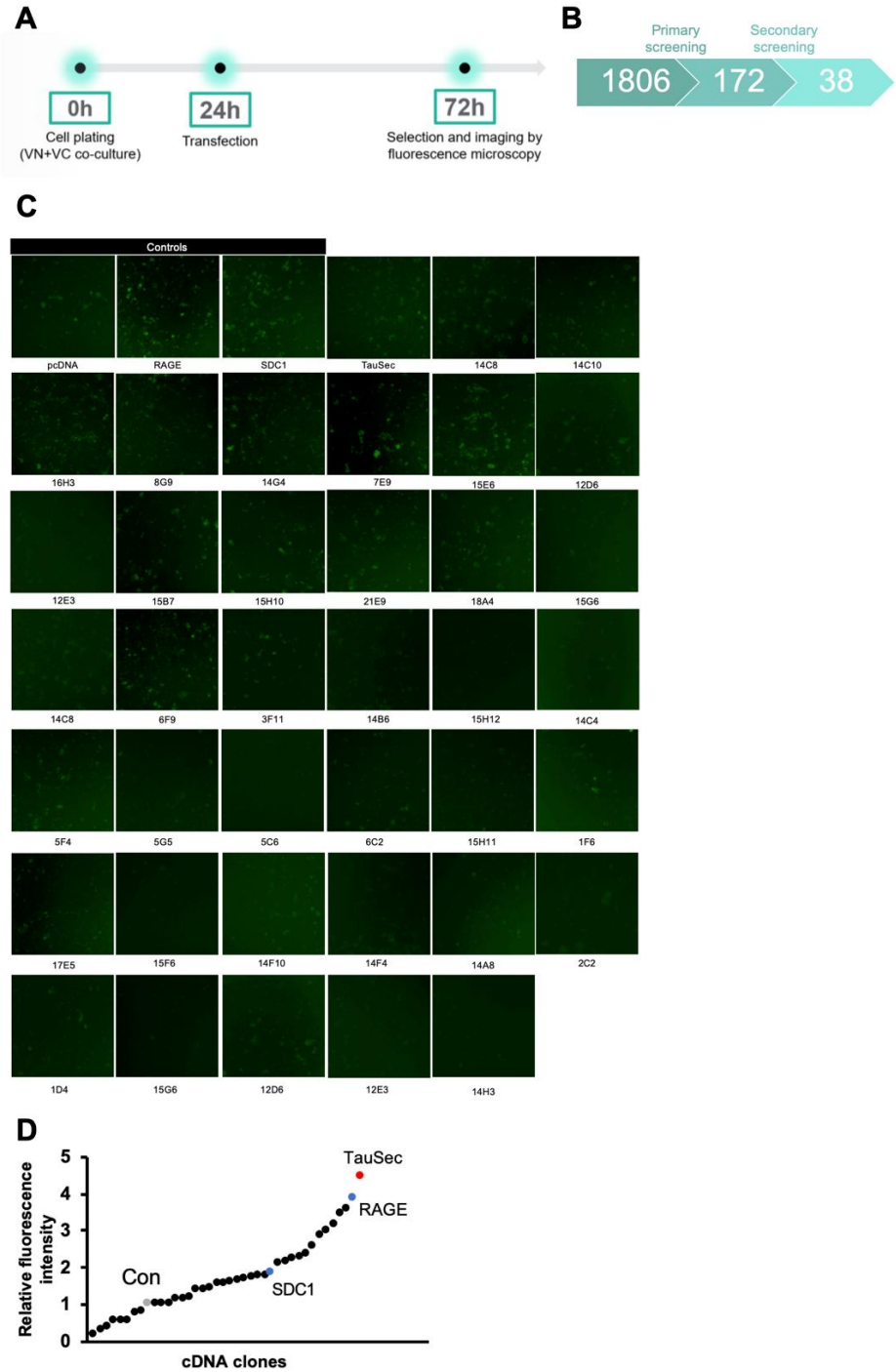

A
0h
Cell plating
(VN+VC co-culture)
24h
Transfection
72h
Selection and imaging by
fluorescence microscopy
B
Primary
screening
Secondary
screening
1806
172
38
C
Controls
pcDNA
RAGE
SDC1
TauSec
14C8
14C10
16H3
8G9
14G4
7E9
15E6
12D6
12E3
15B7
15H10
21E9
18A4
15G6
14C8
6F9
3F11
14B6
15H12
14C4
5F4
5G5
5C6
6C2
15H11
1F6
17E5
15F6
14F10
14F4
14A8
2C2
1D4
15G6
12D6
12E3
14H3
D
Relative fluorescence intensity
cDNA clones
TauSec
RAGE
SDC1
Con
0
1
2
3
4
5

Figure 3. TauSec treatment enhances tau propagation in neuronal cells.

(A) Purification of GST-TauSec protein from *E. coli*.

(B, C) SH-SY5Y-VN and SH-SY5Y-VC cells were co-cultured in the presence of GST-TauSec protein (10 nM) for 48 h and then observed under fluorescence microscope (Red, RFP; green, tau-BiFC) (B). The fluorescence intensity of Tau-BiFC in B was quantified (n = 3). Bars represent mean ± s.e.m.. *p = 0.0279, **p = 0.0010, ****p < 0.0001.

(D) Schematic image of a tau propagation assay using DsRed-IRES-tau EGFP virus. In this assay, viral transduction causes infected cells to emit both red and green signals. When tau propagation occurs to a non-infected cell, only tau-EGFP is transmitted, becoming a DsRed-negative, GFP-positive cell.

(E) Primary cortical neurons (DIV 7) were treated with GST-TauSec (10 nM) for 72 h, increasing the number of RFP-negative and GFP-positive neurons.

(F) Quantification of the numbers of RFP-negative and GFP-positive neurons after treatment with GST-TauSec (n = 3). Bars represent mean ± s.e.m. *p = 0.0319.

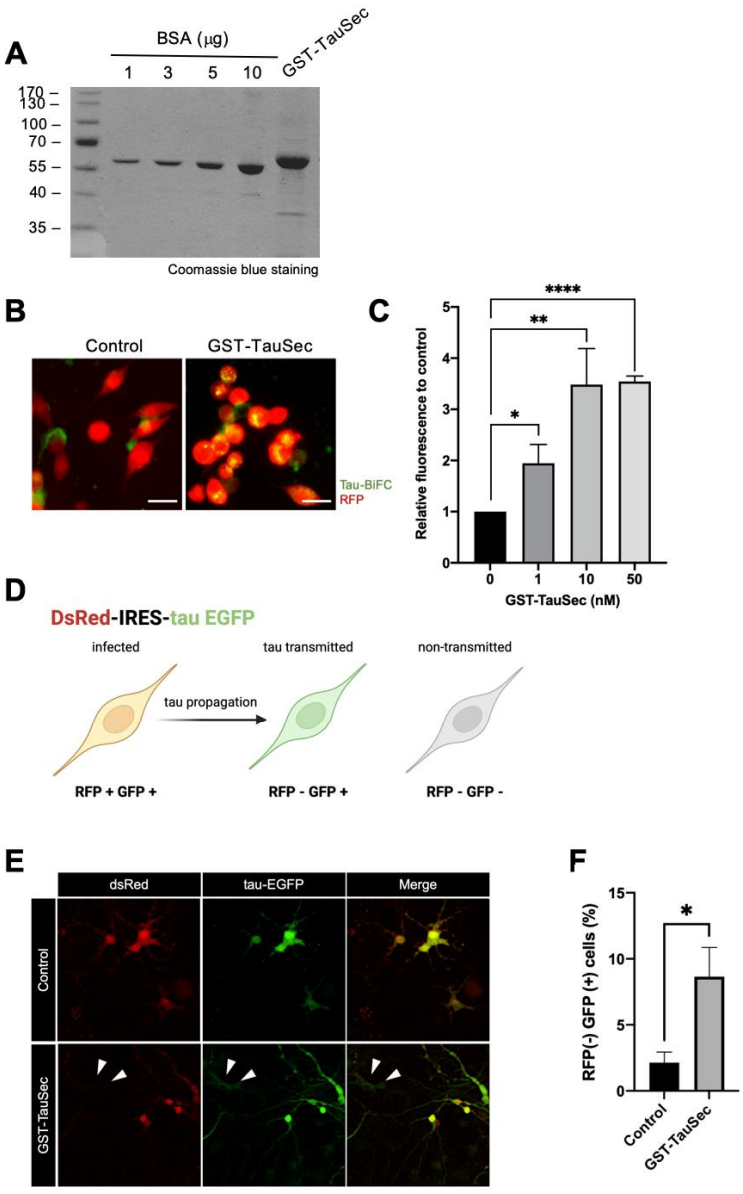

A
BSA (μg)
1
3
5
10
GST-TauSec
170 –
130 –
100 –
70 –
55 –
40 –
35 –
Coomassie blue staining
B
Control
GST-TauSec
Tau-BiFC
RFP
C
****
**
*
Relative fluorescence to control
0
1
2
3
4
5
0
1
10
50
GST-TauSec (nM)
D
DsRed-IRES-tau EGFP
infected
tau transmitted
non-transmitted
tau propagation
RFP + GFP +
RFP - GFP +
RFP - GFP -
E
dsRed
tau-EGFP
Merge
Control
GST-TauSec
F
*
RFP(-) GFP (+) cells (%)
0
5
10
15
Control
GST-TauSec

Figure 4. TauSec promotes tau internalization in SH-SY5Y and mouse primary cortical neurons.

(A) Representative images showing increased internalization of tau-Dylight 488 oligomers [low molecular weight (LMW) and high molecular weight (HMW), 250 nM] within 2 h in SH-SY5Y cells by TauSec (1 nM to 50 nM). (Green, Tau-Dylight 488)

(B) Uptake of tau-Dylight 488 oligomers in (A) was quantified under fluorescence microscope (n = 3). Bars represent mean ± s.e.m. ****p < 0.0001.

(C) TauSec (1 nM, 10 nM) increases cellular uptake of tau-Dylight 488 oligomers (LMW and HMW, 250 nM) in mouse primary cortical neurons after 24 h. (Green, tau; red, MAP2; blue, Hoecht)

(D) Uptake of tau-Dylight 488 oligomers in primary mouse cortical neurons in (A) was quantified (n = 3). Bars represent mean ± s.e.m. **p = 0.0015, ***p = 0.0007.

(E) SH-SY5Y cells were incubated with oligomeric tau (LMW and HMW, 500 nM) in the absence or presence of GST-TauSec (1 nM) and then analyzed by western blotting using HT7 antibody.

(F) SH-SY5Y cells were incubated with tau-Dylight 488 oligomers (LMW and HMW, 250 nM) and GST-TauSec (1 nM) in the absence or presence of Dynasore (40 μM) or EIPA (25 μM).

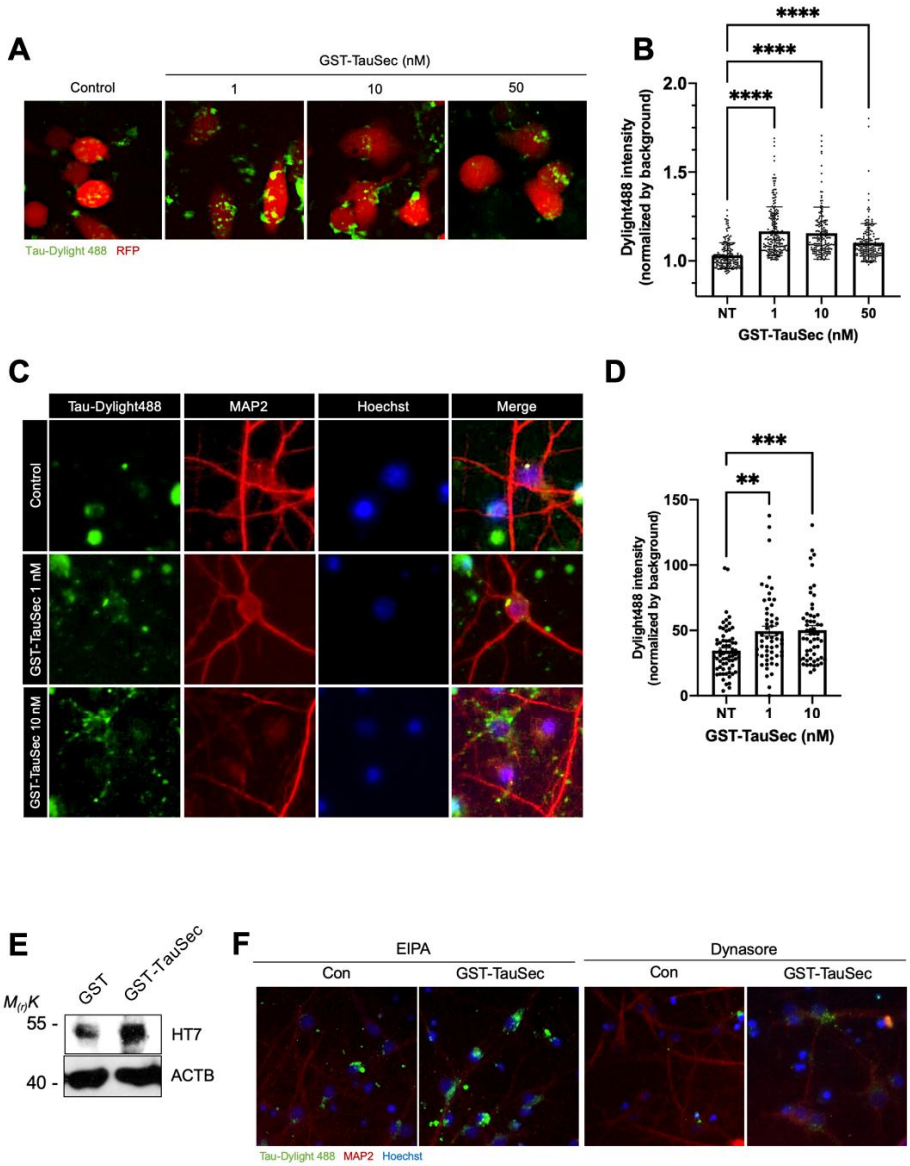

A
GST-TauSec (nM)
Control
1
10
50
Tau-Dylight 488 RFP
B
****
****
****
Dylight488 intensity (normalized by background)
2.0
1.5
1.0
NT
1
10
50
GST-TauSec (nM)
C
Tau-Dylight488
MAP2
Hoechst
Merge
Control
GST-TauSec 1 nM
GST-TauSec 10 nM
D
***
**
Dylight488 intensity (normalized by background)
150
100
50
0
NT
1
10
GST-TauSec (nM)
E
GST
GST-TauSec
M(r)K
55 -
40 -
HT7
ACTB
F
EIPA
Dynasore
Con
GST-TauSec
Con
GST-TauSec
Tau-Dylight 488 MAP2 Hoechst

Figure 5. TauSec expression is increased in primary cortical neurons under various stress conditions.

(A-C) Primary cortical neurons (DIV 7) were treated with amyloid-beta (Aβ) (5 μM), NaCl (1 mM), oligomeric tau (500 nM), or H_2O_2 (100 μM) for 48 h, or transfected with TauSec cDNA, and analyzed with western blotting (A). The signals on the blot were quantified for TauSec expression (n = 3). Bars represent mean ± s.e.m. (B). Total RNAs were purified, analyzed by qRT-PCR and normalized by GAPDH (n = 3). Bars represent mean ± s.e.m. (C).

(D) Mouse primary cortical neurons (DIV 7) were infected with lentivirus-TauSec for 48 h and then analyzed by immunostaining using anti-TauSec and anti-TauSec receptor antibodies (Red, TauSec receptor; green, TauSec; blue, Hoechst). Nuclei were stained with Hoechst dye.

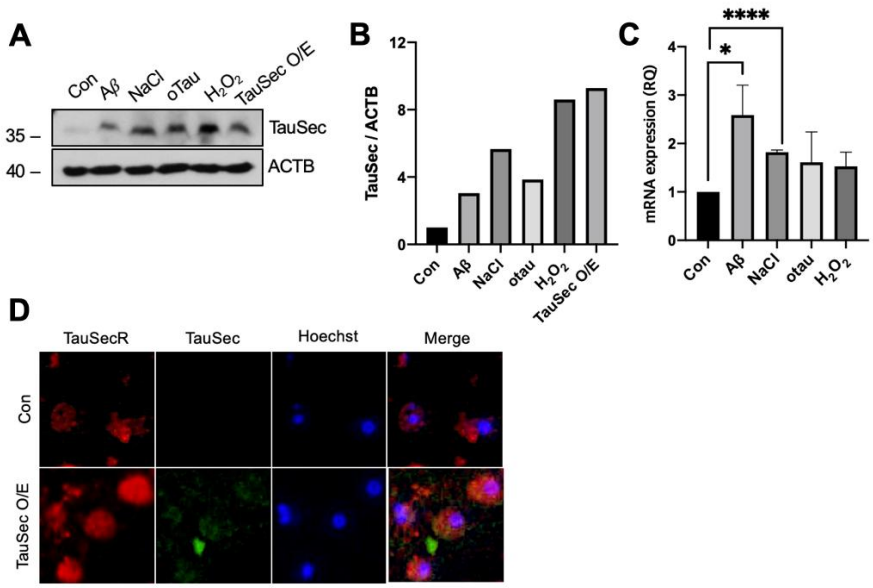

A
Con
Aβ
NaCl
oTau
H_2O_2
TauSec O/E
35 –
40 –
TauSec
ACTB
B
TauSec / ACTB
12
8
4
0
Con
Aβ
NaCl
otau
H_2O_2
TauSec O/E
C
****
*
mRNA expression (RQ)
4
3
2
1
0
Con
Aβ
NaCl
otau
H_2O_2
D
TauSecR
TauSec
Hoechst
Merge
Con
TauSec O/E

Figure 6. TauSec increases the amount and modification of TauSec receptor

(A, B) SH-SY5Y cells were treated with GST-TauSec (1 nM) for indicated periods of time and analyzed by western blotting using anti-TauSec receptor (TauSecR) (A) or anti-ERK antibodies (p-ERK, total ERK) antibody (B).

(C) After treating SH-SY5Y cells with GST-TauSec (1 nM) for 24 h, cell lysates were prepared, treated with PNGase F at 37°C for 1 h and analyzed with western blotting.

(D) SH-SY5Y cells were treated with GST-TauSec (1 nM) for 24 h and total RNA were isolated and analyzed and analyzed by qRT-PCR (n = 3). Bars represent mean ± s.e.m. ns, non=specific; **p = 0.0047.

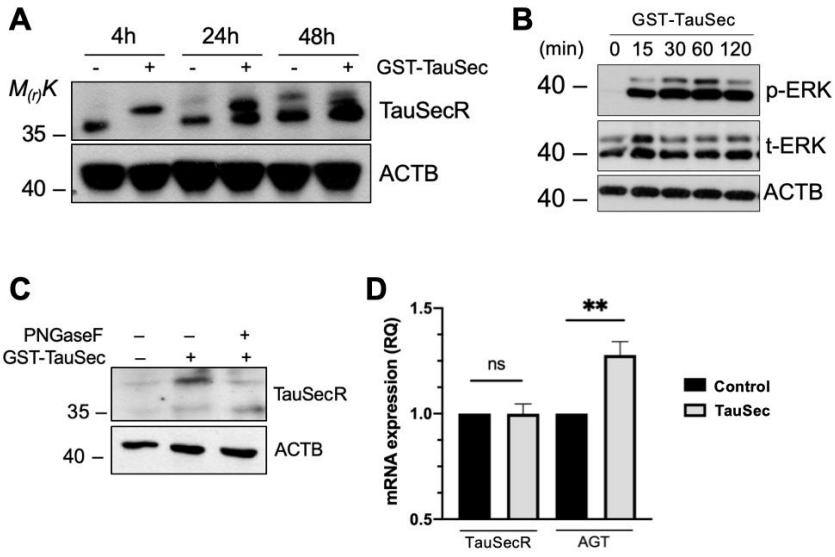

A
4h
24h
48h
- + - + - +
GST-TauSec
$M_{(r)}K$
35 –
40 –
TauSecR
ACTB
B
GST-TauSec
(min) 0 15 30 60 120
40 –
p-ERK
40 –
t-ERK
40 –
ACTB
C
PNGaseF – – +
GST-TauSec – + +
35 –
TauSecR
40 –
ACTB
D
mRNA expression (RQ)
1.5
1.0
0.5
ns
**
Control
TauSec
TauSecR
AGT

Figure 7. TauSec receptor is essential in TauSec-mediated tau internalization

(A) SH-SY5Y/TauSec receptor knockdown (sgTauSecR#1, 2 and 3) cell lines generated via CRISPR-Cas9 system using 3 different sgRNAs were analyzed by western blotting.

(B) SH-SY5Y/sgTauSecR#1, 2 and 3 cells were incubated with tau-Dylight 488 oligomers (LMW and HMW, 250 nM) for 2 h and then observed for tau uptake under fluorescence microscope. Bars represent mean ± s.e.m.*p = 0.0182, **p = 0.0053, ***p = 0.0005.

(C) SH-SY5Y/sgControl (sgCtrl) and SH-SY5Y/sgTauSecR #3 cells were treated for 2 h with tau-Dylight 488 oligomers (LMW and HMW, 250 nM) with or without GST-TauSec (1 nM) and then observed under a fluorescence microscope (Red, RFP; green, tau-Dylight 488)

(D, E) Mouse primary cortical neurons were infected with lentivirus with TauSec receptor shRNA#1 and 2 and analyzed by western blotting (D) or incubated for 12 h with pH Rhodo-tau oligomers (LMW and HMW, 500nM) with or without TauSec (1 nM). Fluorescence intensity of intraneuronal tau was observed under fluorescence microscope (F) and quantified (E). Bars represent mean ± s.e.m. ****p < 0.0001.

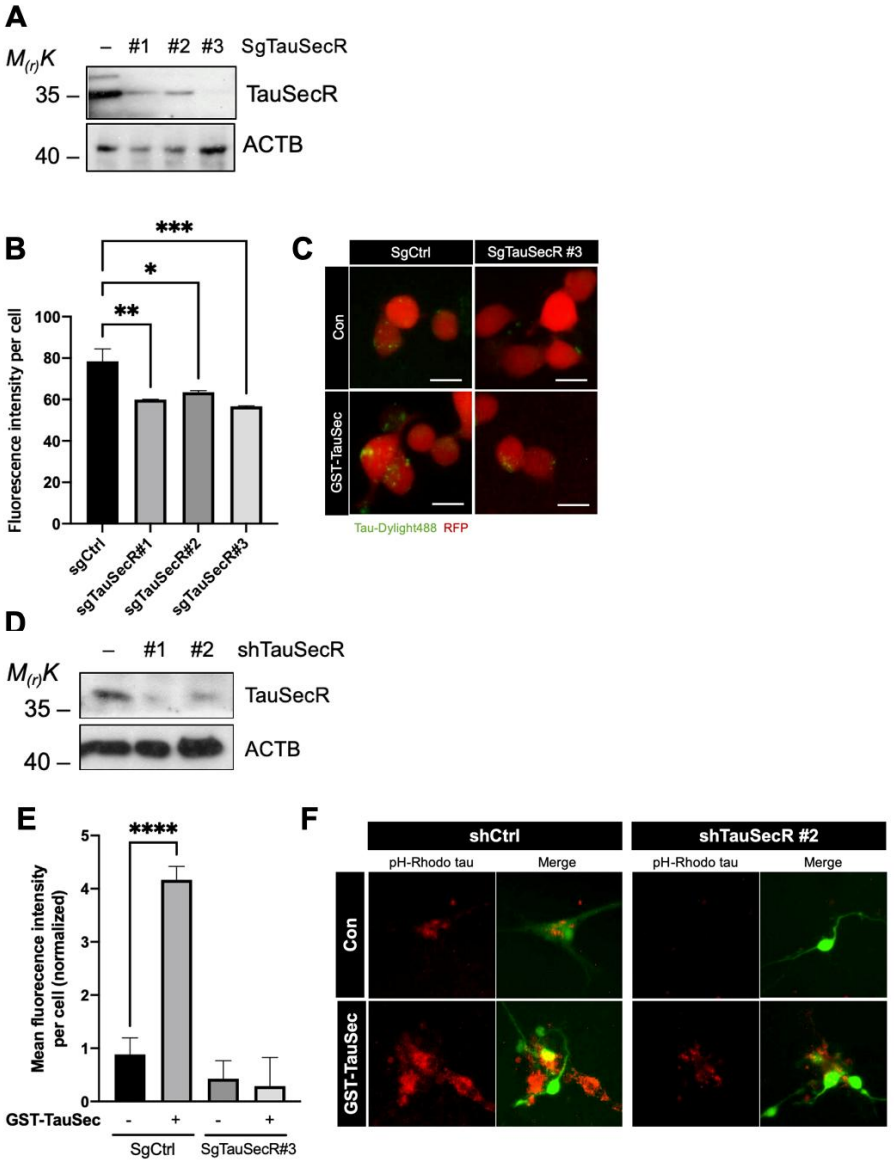

A
– #1 #2 #3 SgTauSecR
$M_{(r)}K$
35 –
TauSecR
40 –
ACTB
B
***
*
**
Fluorescence intensity per cell
100
80
60
40
20
0
sgCtrl
sgTauSecR#1
sgTauSecR#2
sgTauSecR#3
C
SgCtrl
SgTauSecR #3
Con
GST-TauSec
Tau-Dylight488 RFP
D
– #1 #2 shTauSecR
$M_{(r)}K$
35 –
TauSecR
40 –
ACTB
E
****
Mean fluorecence intensity per cell (normalized)
5
4
3
2
1
0
GST-TauSec - + - +
SgCtrl
SgTauSecR#3
F
shCtrl
shTauSecR #2
pH-Rhodo tau
Merge
pH-Rhodo tau
Merge
Con
GST-TauSec

Figure 8. TauSec-TauSec receptor interaction is important for tau uptake than TauSec's protease activity

(A) The primary structure of TauSec. Pre and pro-segment are indicated. Numbers indicate amino acid sequence of human TauSec. The pro-segment of TauSec can aid in receptor binding and inhibit substrate binding.

(B) GST-proTauSec and active TauSec lacking the pro-segment were purified from *E. coli* and analyzed by western blotting.

(C, D) SH-SY5Y cells were incubated for 2 h with tau-Dylight 488 oligomers (LMW and HMW, 250 nM) in the presence or absence of GST-proTauSec (1 nM) or GST-active TauSec (1 nM) and then observed under fluorescence microscope (C). The fluorescence intensity in the images (C) was quantified and represented as bars (mean ± s.e.m.). ns, nonspecific; ****$p < 0.0001$ (D).

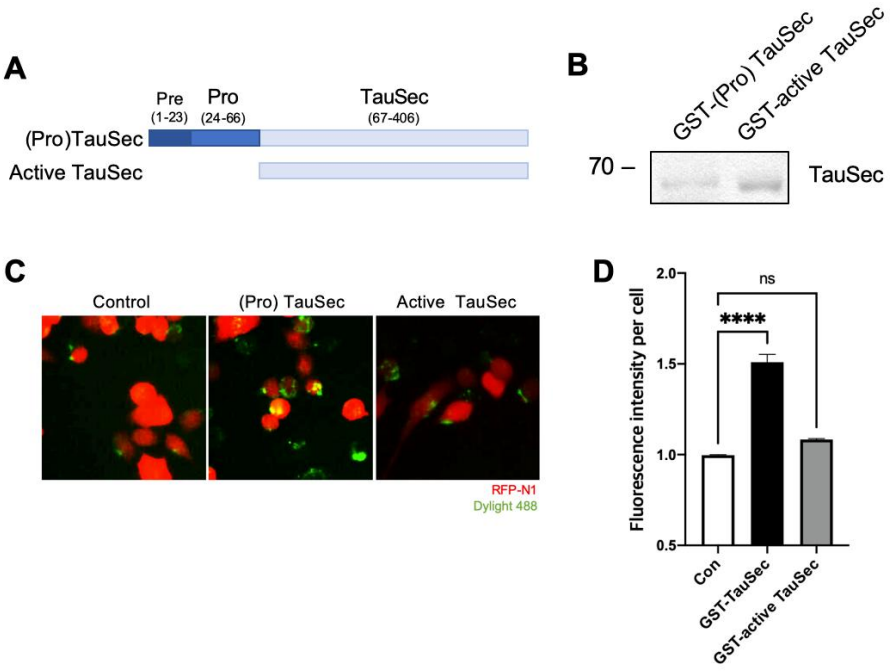

A
Pre
(1-23)
Pro
(24-66)
TauSec
(67-406)
(Pro)TauSec
Active TauSec
B
GST-(Pro) TauSec
GST-active TauSec
70 –
TauSec
C
Control
(Pro) TauSec
Active TauSec
RFP-N1
Dylight 488
D
Fluorescence intensity per cell
2.0
1.5
1.0
0.5
ns
****
Con
GST-TauSec
GST-active TauSec

Figure 9. Prolonged exposure to TauSec causes tau accumulation in neuronal cells

(A, B) Mouse primary cortical neurons were left untreated or exposed to GST-TauSec (10 nM) for 48 h and analyzed by western blotting (A) and the signals of DA9 on the blots were quantified and normalized to beta-actin (ACTB) (n = 3). Bars represent mean ± s.e.m. *p = 0.0214 (B).

(C) SH-SY5Y/tau-VC cells are incubated with TauSec (1 nM), bafilomycin (10 nM) or MG132 (5 μM) for the indicated times and then analyzed by western blotting. Signals of HT7 on the blots were quantified and normalized to beta-actin (ACTB) (n = 3). Bars represent mean ± s.e.m. **p = 0.0086 (D).

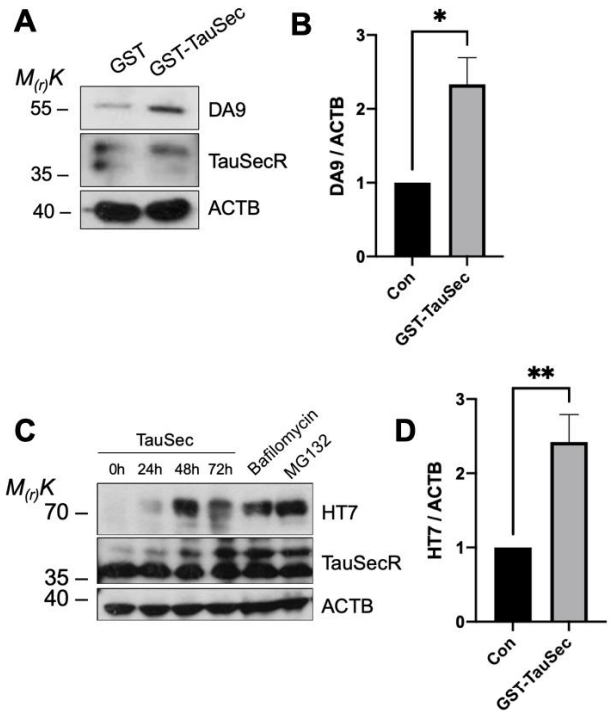

A
GST
GST-TauSec
M(r)K
55 –
35 –
40 –
DA9
TauSecR
ACTB
B
*
DA9 / ACTB
3
2
1
0
Con
GST-TauSec
C
TauSec
0h 24h 48h 72h
Bafilomycin
MG132
M(r)K
70 –
35 –
40 –
HT7
TauSecR
ACTB
D
**
HT7 / ACTB
3
2
1
0
Con
GST-TauSec

Figure 10. TauSec increases lysosomal pH.

(A, B) SH-SY5Y and SH-SY5Y/sgTauSecR#3 cells expressing pHLare were treated with or without GST-TauSec (10 nM) for 24 h and then observed under fluorescence microscope (A). The fluorescence intensity of the images was quantified, and pH was calculated as a ratio of sfGFP to mCherry. Bars represent mean ± s.e.m. (B)

(C, D) SH-SY5Y cells were left untreated or incubated with TauSec (10 nM) for 48 h in the presence of DQ-Red BSA (12 μg/mL) (C) and fluorescence intensity of the images was quantified (B). Bars represent mean ± s.e.m. **p = 0.0043.

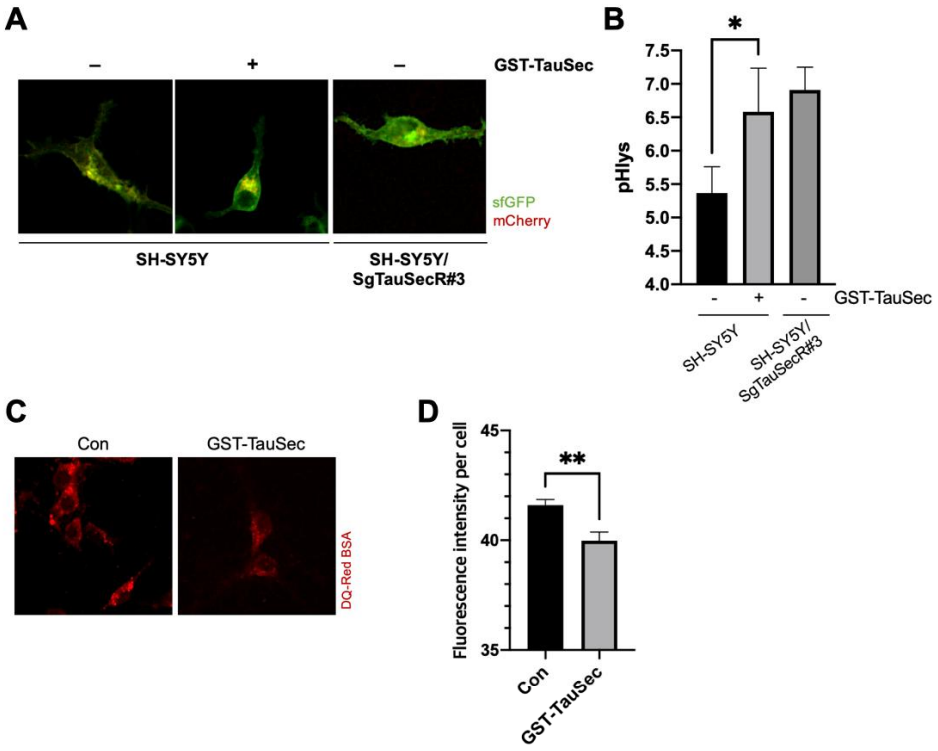

A
–
+
–
GST-TauSec
sfGFP
mCherry
SH-SY5Y
SH-SY5Y/
SgTauSecR#3
B
*
pHlys
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
-
+
-
GST-TauSec
SH-SY5Y
SH-SY5Y/
SgTauSecR#3
C
Con
GST-TauSec
DQ-Red BSA
D
**
Fluorescence intensity per cell
45
40
35
Con
GST-TauSec

Figure 11. Anti-TauSec receptor antibody blocks TauSec-mediated tau uptake.

(A) SH-SY5Y cells were treated with GST-TauSec (10 nM) for 30 min in the presence or absence of anti-TauSec receptor antibody (5 and 50 μg/mL, ABclonal Science, Inc.) and then analyzed by western blotting.

(B, C) SH-SY5Y cells were treated for 2 h with GST-TauSec (10 nM) and tau-Dylight 488 oligomers (LMW and HMW, 250 nM) with or without anti-TauSec receptor antibody (5 and 50 μg/mL) and then observed under fluorescence microscope (B). The fluorescence intensity of the images was quantified and represented as bars (mean ± s.e.m.). ****$p < 0.0001$ (C).

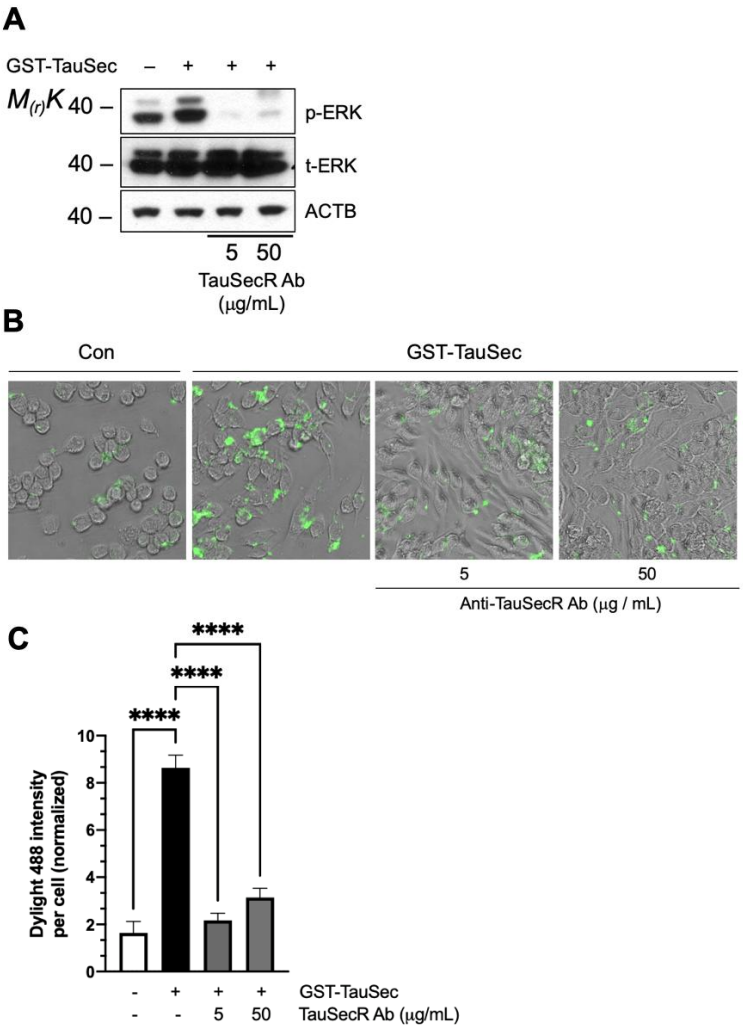

A
GST-TauSec – + + +
M(r)K 40 –
40 –
40 –
p-ERK
t-ERK
ACTB
5 50
TauSecR Ab
(μg/mL)
B
Con
GST-TauSec
5
50
Anti-TauSecR Ab (μg / mL)
C
****
****
****
Dylight 488 intensity
per cell (normalized)
10
8
6
4
2
0
- + + + GST-TauSec
- - 5 50 TauSecR Ab (μg/mL)

Figure 12. TauSec/TauSec receptor PPI compound inhibits the interaction between TauSec and TauSec receptor

(A) GST-TauSec and His-TauSec receptor ectodomain (a.a. 17 - 302) proteins were purified and resolved in SDS-PAGE.

(B) Purified His-TauSec receptor ectodomain (ecto) and SH-SY5Y cell lysate expressing endogenous full-length (FL) TauSec receptor were detected by anti-TauSec receptor antibody by western blotting.

(B) His-TauSec receptor ectodomain (100 nM) were attached on the plate and then overlayed with GST-TauSec (10 nM) in the presence of RR-PPI. The interaction between GST-TauSec and His-TauSec receptor ectodomain was measured by observance at 450 nM (n = 3).

(C) Live-cell ELISA with or without RR-PPI drug (10 μM) in SH-SY5Y. Cells were incubated with RR-PPI drug and GST-TauSec (10 nM) on ice for 10 min. GST antibody was added to measure binding, and fluorescence intensity was measured. *$p < 0.0001$.

(D) Live-cell ELISA with or without RR-PPI drug (10 μM) in SH-SY5Y/SgTauSecR#3 where WT TauSec receptor was overexpressed. Cells were incubated with RR-PPI drug and GST-TauSec (10nM) on ice for 10 min. GST antibody was added to measure binding, and fluorescence intensity was measured. *$p < 0.0001$.

(E) SH-SY5Y cells were treated with TauSec (1 nM) in the presence

or absence of the indicated concentrations of RRPPI and analyzed by western blotting using anti-ERK and anti-pERK antibodies.

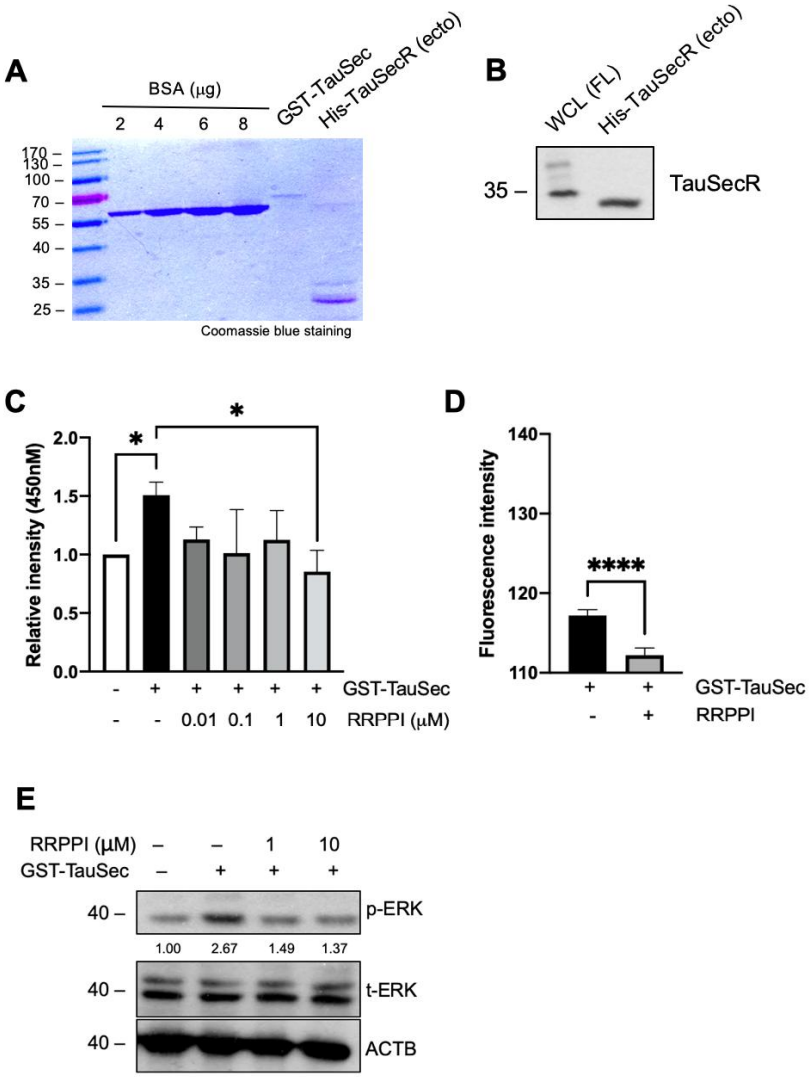

A
BSA (μg)
2 4 6 8
GST-TauSec
His-TauSecR (ecto)
170 –
130 –
100 –
70 –
55 –
40 –
35 –
25 –
Coomassie blue staining
B
WCL (FL)
His-TauSecR (ecto)
35 –
TauSecR
C
Relative inensity (450nM)
2.0
1.5
1.0
0.5
0.0
*
*
- + + + + + GST-TauSec
- - 0.01 0.1 1 10 RRPPI (μM)
D
Fluorescence intensity
140
130
120
110
****
+ + GST-TauSec
- + RRPPI
E
RRPPI (μM) – – 1 10
GST-TauSec – + + +
40 –
p-ERK
1.00 2.67 1.49 1.37
40 –
t-ERK
40 –
ACTB

Figure 13. TauSec/TauSec receptor-PPI inhibits TauSec-mediated tau propagation and accumulation.

(A) SH-SY5Y-VN/SH-SY5Y-VC cells were co-cultured in the presence or absence of RR-PPI (0.1 and 1 μM) for 48 h and then observed under fluorescence microscope. Bars represent mean ± s.e.m. ****$p < 0.0001$.

(B, C) SH-SY5Y/tau-VC cells were incubated with GST-TauSec (1 nM) and the indicated concentrations of RR-PPI for 24 h and analyzed with western blotting (B). The signals on the blots were quantified by densitometric analysis. Bars represent mean ± s.e.m. *$p = 0.0072$, 0.0029, 0.0084 respectively (C).

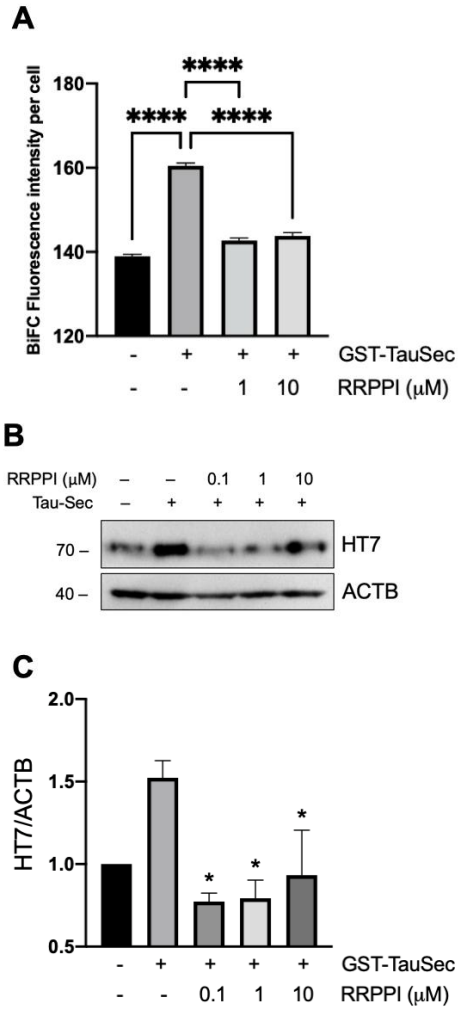
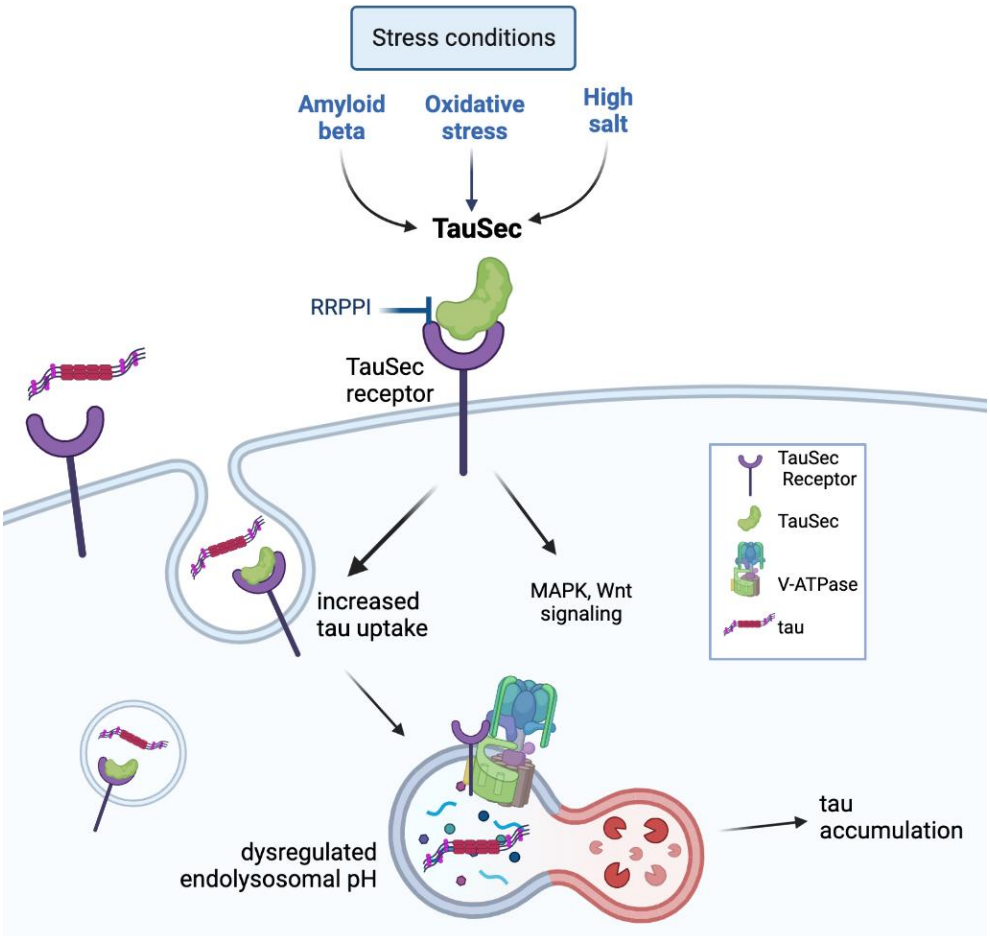

A
BiFC Fluorescence intensity per cell
180
160
140
120
****
****
****
- + + + GST-TauSec
- - 1 10 RRPPI (μM)
B
RRPPI (μM) – – 0.1 1 10
Tau-Sec – + + + +
70 –
40 –
HT7
ACTB
C
HT7/ACTB
2.0
1.5
1.0
0.5
*
*
*
- + + + + GST-TauSec
- - 0.1 1 10 RRPPI (μM)

Figure 14. Schematic image of the role of TauSec in tau propagation

TauSec is upregulated under stress conditions such as Amyloid beta, oxidative stress, and high salt conditions. TauSec interacts with the TauSec receptor, which can be blocked by RRPPI drug. The interaction between TauSec and TauSec receptor initiates downstream signaling pathways such as ERK or Wnt signaling, and it can also increase tau uptake.

As a V-type ATPase accessory protein, the TauSec receptor is associated with vesicle acidification. When exposed to increased amounts of TauSec, the TauSec receptor is dysregulated, impairing endolysosomal pH, which leads to intracellular tau accumulation.


Stress conditions
Amyloid beta
Oxidative stress
High salt
TauSec
RRPPI
TauSec receptor
increased tau uptake
MAPK, Wnt signaling
TauSec Receptor
TauSec
V-ATPase
tau
dysregulated endolysosomal pH
tau accumulation

DISCUSSION

Despite active research in the field of tau propagation, relatively little is known about its underlying mechanism and methods to alleviate it. Because tau propagation is essential in tau pathology and the development of Alzheimer's Disease (AD), I screened over 1,800 genes to identify potential tau propagation modulator using the tau-BiFC propagation assay. While the tau-BiFC propagation assay is an excellent system to monitor and test tau propagation in cells, the overall fluorescence intensity is fairly low. Therefore, potential promoters of tau propagation were easily identified, whereas potential inhibitors of tau propagation were difficult to find. While finding a gene that can potentially inhibit tau propagation would have been helpful, finding TauSec as a tau propagation promoter gene is significant because understanding the underlying mechanism can elucidate the link between hypertension and AD. This suggests bright prospects for AD therapy, as various anti-hypertensive drugs targeting TauSec and the RAS pathway can be applied to AD therapy.

TauSec increased tau uptake and accumulation in neuronal cells by interacting with the TauSec receptor. Interaction between TauSec and the TauSec receptor activated the TauSec receptor and

caused accumulation and modification of the TauSec receptor, and depletion of the TauSec receptor inhibited tau internalization. TauSec receptor is known to be essential in the assembly of V-type ATPase, and it has been found to play a significant role in receptor-mediated endocytosis and lysosomal function in the renal proximal tubule (Figueiredo et al., 2021). These findings suggest that dysregulation of the TauSec receptor caused by excessive TauSec exposure can affect the endolysosomal pathway of tau internalization and processing in neuronal cells. This hypothesis is supported by the evident increase in lysosomal pH in SH-SY5Y when GST-TauSec is treated. Increased internalization and the failure to degrade tau caused by TauSec seems to promote cell-to-cell tau propagation.

While little is known about brain TauSec, I have discovered that the expression of TauSec, albeit in low levels, is increased in response to stress conditions. In high sodium culture condition that mimics chronic hypertension and even AD, neuronal TauSec expression is increased. Similarly, in other stress conditions such as oxidative stress, Aβ, and tau oligomer treatment, TauSec is upregulated. It can be said that the upregulation of TauSec under stress conditions that are often found in aged or hypertensive brains can promote tau propagation, exacerbating AD. This finding is significant in that it explains the relationship between RAS and

AD in terms of tau propagation rather than through microbleeding or microinfarcts, lesions common in VD.

In vivo experiments must be conducted in order to confirm the effect of TauSec in tau propagation in AD pathology. TauSec's role in promoting tau propagation can be tested by overexpressing TauSec in AD model mice, such as PS19. Because TauSec cannot cross the BBB, overexpressing TauSec in the brain seems to be the most plausible way to observe TauSec's effect in the brain. TauSec-overexpressing mice should be tested for their memory and behavior by Y-maze and novel object recognition test. Moreover, through immunohistochemical analysis of their brains, changes in TauSec receptor levels, tau pathology, and most importantly, tau propagation, should be observed.

For patient relevance, it would be ideal to obtain human patient brain samples and observe the amount of TauSec and TauSec receptor in patient brains via immunohistochemistry or western blotting. Currently, brain organoids made by human patient-derived iPSC cells are being tested to confirm the effect of TauSec by treating purified GST-TauSec and overexpressing TauSec by viral transduction. If TauSec does induce tau propagation and pathology in such condition that more closely resembles the physiological human brain, it will greatly add to the

significance and reliability of this study.

These findings reveal a potential tau propagation modulator that could be a link between hypertension and AD. This has important implications for AD therapeutics. Given the low expression of TauSec, effects of TauSec in in vivo settings and the scope of TauSec's role in overall tau pathology and propagation in human patients remain to be studied. This study suggests a possible explanation for the correlation between hypertension and AD and enhances our understanding of how tau is internalized and processed in neuron-to-neuron propagation.

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국문 초록

Tau 확산 (propagation)은 알츠하이머병 (AD) 환자의 뇌에서 비정상적 Tau가 세포 간 전파가 되면서 마치 prion처럼 정상적인 tau의 응집을 유도함으로써 진행된다. Tau propagation은 AD 진행에 핵심적인 단계이나 그 기전이 규명되지 않았다. 따라서 본 연구에서 tau propagation 조절 인자 발굴을 통해 기전을 설명하고자 한다. Tau propagation의 조절 인자를 찾기 위해 tau-BiFC propagation assay를 사용하여 1,800여개의 secretory protein 유전자를 screening 하였다. Tau-BiFC propagation assay는 tau propagation을 쉽게 관찰할 수 있는 cell-based assay로서 tau propagation이 일어난 세포에서만 형광이 관찰되어 tau propagation 발생 정도를 수치적으로 측정 가능하다.

스크리닝을 통해 TauSec 유전자가 tau propagation을 증가시키는 유전자로 동정되었다. TauSec은 tau propagation의 복합적인 과정 중에서 특히 uptake 단계를 촉진시키는 것으로 관찰되었다. 정제된 TauSec 단백질과 tau oligomer 처리 시 초기에 SH-SY5Y cell과 mouse primary cortical neuron에서 모두 tau uptake가 증가하는 것을 관찰할 수 있었다.

TauSec에 의한 tau uptake에는 TauSec과 TauSec receptor의 상호작용이 핵심적으로 보인다. TauSec receptor을 knock-down한 SH-SY5Y와 mouse primary cortical neuron에서 tau uptake가 현저히

감소했으며 TauSec protein을 처리해도 tau uptake를 증가시키지 못하였다. TauSec 처리 시 세포 내 TauSec receptor의 양과 modification 이 증가하는데 발현이 증가하는 것이 아니라 TauSec receptor 단백질이 축적되는 것으로 확인된다. TauSec receptor은 endolysosomal pathway에 관여하는 단백질로 알려져 있으며 장시간 TauSec 처리 시 과도한 modification이 일어나면서 정상적인 기능을 하지 못해 lysosome의 기능에 악영향을 끼치는 것으로 보인다. 따라서 TauSec은 후기에 tau clearance를 저해하는 역할까지 함으로서 tau propagation을 악화시킨다는 결론을 내릴 수 있다.

Tau propagation은 임상 치료에서 큰 의미를 가진다. Tau propagation 조절 인자를 저해하는 약물이나 항체가 개발된다면 독성을 가진 tau가 전파되기 전에 단백질을 제어할 수 있을 것이다. Tau propagation이 대뇌의 대부분 영역으로 확산되면 신경세포 사멸로 이어지기 때문에 그 단계를 방지하는 것이 치매 치료에 핵심적일 것이다. 뉴런에서 응집된 단백질의 확산은 tau 뿐만 아니라 파킨슨병(PD)에서 나타나는 alpha-synuclein에서도 보이는 현상이다. 따라서 향후에 본 연구가 PD 치료에까지 응용될 가능성도 있다.

주요어 : 알츠하이머병, Tauopathy, 2분자 형광 상보성 (Bimolecular Fluorescence Complementation), Tau 확산, Tau 세포 내 유입

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