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Modeling endophilin-mediated Aβ disposal in glioma cells

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ABSTRACT

Autophagy dysregulation has emerged in age-related neurological diseases (Ulland et al.; Matheoud et al.; Ashkenazi et al.). Alzheimer Disease (AD), the most common progressive neurodegenerative disorder, is characterized by the accumulation of amyloid-β (Aβ) plaques caused by aberrant Aβ metabolism (Qiang et al.; Sevigny et al.; Ittner et al.). Glia constitute the brain immune system and ingest extracellular Aβ for degradation via the autophagy-lysosome machinery (Ries and Sastre; Cho et al.). Here, we model the molecular rationale for this clearance process in glioma cells by showing that miR34a inhibits autophagy-mediated disposal of Aβ fibrils and identifying two novel direct targets of miR34a, endophilin-3 and cathepsin B (CTSB, a previously reported enzyme for Aβ degrading (Sun et al.)). Bioinformatics analyses revealed that endophilin-3 expresses at a significantly lower level in neurodegenerative diseases. Its gain-of-function substantially promotes both uptake and degradation of Aβ while small interfering RNA (siRNA)-mediated endophilin-3 knockdown slowed down Aβ clearance and blocked autolysosome formation. Mechanistically, gene ontology (GO) analysis of the endophilin-3 interactome identified by mass spectrometry uncovered enriched components involved in actin binding (with the highest score). Importantly, we validated that the actin-binding protein phostensin interacted with endophilin-3. Phostensin knockdown restored endophilin-3-mediated up-regulation of Aβ clearance. Thus, our findings indicate that miR34a inhibits Aβ clearance by targeting endophilin-3 and CTSB at multiple steps including uptake and autophagy-mediated degradation.

1. Introduction

Autophagy is an evolutionarily conserved proteostasis and catabolic membrane trafficking process that has close associations with health and longevity [1–3, 10, 11]. It maintains cellular homeostasis and viability by regulating the quantity and quality of cytoplasmic constituents such as organelles and macromolecules through lysosomal degradation, which is activated in response to nutrient starvation and other stressors to generate energy and allow survival [12]. Autophagy plays vital roles by self-degrading under a variety of physiological and pathological conditions such as maintaining the stem cell quiescent state [13], manipulating immunity [14], regulating bone growth [15] and carcinogenesis [16, 17]. Importantly, autophagy directly targets pathogenic misfolded Aβ for degradation [6, 18]. Chemical that enhances autophagy was shown to be capable of restoring nesting behavior in a murine model of AD [19]. In addition to the extensive efforts have been made on Aβ degradation by autophagy, it was also argued that autophagy activity could be modulated by SUMO1 for Aβ genesis [20]. Despite that TRPM7 activation increases basal autophagy and promote Aβ degradation in vitro [21], autophagy induced by fasting in AD mouse model seemed not to sufficiently degrade Aβ [22], suggesting that some downstream events may be blocked which thus impedes autophagy-mediated Aβ clearance.

The BIN/amphiphysin/Rvs (BAR) domain-containing protein endophilins are a group of proteins highly responsible for the fast-acting tubulovesicular endocytosis [23, 24], during which endophilins are promoted by polyunsaturated phospholipids to deform and vesiculate synthetic membranes [25]. The endophilin family consists of five members: endophilin-1, endophilin-2, endophilin-3, endophilin-B1 and endophilin-B2. Some of these have been noticed in recent neuroscience research [26, 27]. CPG2 directly interacts and recruits endophilin-B2 to F-actin to facilitate glutamate receptor internalization at synapse [28].

Abbreviations: AD, Alzheimer disease; Aβ, amyloid-β; GO, gene ontology; LC3, microtubule-associated protein 1A/1B-light chain 3; WT, wide-type; CTSB, cathepsin B; WB, Western blot; ELISA, Intracellular enzyme-linked immunosorbent assay; UTR, untranslated region; GEO, Gene Expression Omnibus; MS, mass spectrometry; XBR, X2 box repressor; HA, hemagglutinin; PCC, Pearson correlation coefficient

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Notably, endophilin-B1 can promote mitochondrial elongation [29] in neurons, and its loss exacerbates AD pathology [30]. Nonetheless, the lack of cellular and molecular studies has precluded a clear understanding of endophilin roles in neurology. Furthermore, few endophilins have been ascribed function in Aβ clearance.

MicroRNAs (miRNAs) are powerful regulators in both AD pathology [31] and autophagy [32, 33]. In agreement with previous publications, we determined that miR34a expressed at a significantly higher level in the hippocampus of a 10-month old (an age that these mice showed typical AD phenotypes [34]) APPswe/PSΔE9 AD mouse model compared to the age-matched control (Supplementary Fig. 1A). Consistently, miR34a also increases in peripheral blood mononuclear cells of AD patients [35]. Recent epigenomic studies have highlighted the immune processes in AD predisposition [36]. Accordingly, immunotherapy against Aβ to trigger its clearance has been tried for AD treatment [5, 37]. Glia constitute about half of the cells in the central nervous system and maintain brain immunity and homeostasis, while astrocytes make up the largest glial population. Abnormal astrocyte function disrupts neuronal morphology associated with AD [7, 38]. In this study, we aimed to elucidate whether miR34a is involved in participating in Aβ clearance and further model the underlying molecular rationale in glioma cells. Key questions to be addressed regarding the roles of miR34a in Aβ clearance concern the mechanisms by which miR34a interfaces with the autophagy-lysosome pathway. We show that miR34a inhibits autophagy-mediated disposal of Aβ in the cells by directly targeting endophilin-3 and CTSB. Furthermore, gain-of- and loss-of-function studies proved that endophilin-3 is a prominent mediator for miR34a in Aβ uptake and degradation. GO analysis of the endophilin-3 interacting proteins revealed a group of highly enriched components involved in actin binding. Identification of endophilin-3-phostensin interaction mechanistically explains how the miR34a-endophilin-3 axis accounts for Aβ clearance.

2. Materials and methods

2.1. Transgenic mice, care and handling

Double transgenic mice express a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9) were purchased from The Jackson Laboratory (004462, Bar Harbor, ME, USA). All animal treatments were approved by the Animal Ethics Committee of Tsinghua University. Mice had access to pre-filtered sterile water and standard mouse chow and were housed under a reversed day-night rhythm in accordance to local legislation on animal welfare.

2.2. Cell lines and reagents

A172 and U251 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and the Chinese Academy of Sciences (Kunming, Yunnan, China), respectively. Cell cultures were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Carlsbad, CA, USA) in a standard incubator at 37 °C with 5% CO_2. Aβ42 was purchased from Millipore (Darmstadt, Germany) and fluorescein isothiocyanate (FITC)-Aβ42 was from AnaSpec (Sacramento, CA, USA). E64D was purchased from Sigma (St. Louis, MO, USA), bafilomycin A1 (Baf, 11038) from Cayman Chemical (Ann Arbor, Michigan, USA) and LysoTracker Red DND-99 from Thermo Fisher Scientific.

2.3. Preparation of Aβ fibrils

Aβ fibrils were prepared as previously described [4, 8]. Briefly, Aβ1-42 was dissolved in DMSO to a final concentration of 500 μM. DMEM-F12 with 10% FBS was added to bring the peptides to a final concentration of 50 μM. Aβ was then incubated for 24 h at 37 °C.

2.4. Establishing stable cell lines

For the miR34a cell line, we employed the cytomegalovirus (CMV) promoter in the GV342 vector (Genechem, Guangdong, China) to express pre-miR34a. Pre-miR34a was cloned with the forward primer 5′-CCAACTTTGTGCCAACCGGATCCTTTCTTTCCTCCCCACATTTC-3′ and reverse primer 5′-CACACATTCCACAGGAATTCGCAGAAGAGCTTC CGAAGTCC-3′. The empty vector was used as the control. 48 h after lentivirus infection, cells were selected by 1 μg/ml puromycin (Thermo Fisher Scientific). For endophilin-3 overexpression, the gene was cloned from the SH3GL3 cDNA (TAKARA, Kusatsu, Shiga, Japan) with the forward primer 5′-GCGCTAGCATGGATGGAATCTTTGCTGGGATAATA TGCAATC-3′, and reverse primer 5′-GCAAGCTTAAGCGTAATCTGGAA CATCGTATGGGTACTGAGGTAAAGGCAC-3′ and subcloned into the pcDNA3.1 vector (Thermo Fisher Scientific) between the *Nhe*I and *Hin*dIII sites. Transfection and selection was pursuant to previous description [39]. The empty vector served as the negative control. For the EGFP-LC3 cell line, the pEGFP-LC3 overexpression plasmid was previously prepared in our lab and used for cell transfection, followed by G418 (100 μg/ml) selection.

2.5. Transient transfection

Cells were transfected with the miR34a, an antisense miR34a inhibitor (anti-miR34a), si*SH3GL3* (5′-GUAAUGCAUUGAUAGAAGU-3′), si*CTSB* (5′-AGAGAGUUAUGUUUACCGA-3′), si*SH3GL2* (5′-GAACCAA GCUAGAUGAUGA-3′), si*SH3GL1* (5′-GCAAGGCGGUGACAGAAGU-3′), and si*PPP1R18* (5′-GCUCGAGAAUGGACACCCA-3′), using Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific) according to the manufacture's recommendation. A scramble siRNA (5′-UUCUCCGAACGUGUCACGUTT-3′) was used as negative control. 48 or 72 h after transfection, cells were treated as indicated in the figure legends.

2.6. Western blot (WB)

Cells were lysed with Laemmli buffer, and each lysate sample was loaded into a polyacrylamide gel and separated by electrophoresis at 30 mA following by transfer onto PVDF membranes (Millipore). Membranes were blocked for 1 h at room temperature using 5% skim milk in Tris-Buffered Saline with 0.1% Tween-20 (TBST). WB analysis was performed according to the antibody manufacturer's specifications. Then, membranes were incubated with primary antibodies overnight in 5% skim milk in TBST at 4 °C. The appropriate horseradish peroxidase (HRP)-conjugated secondary antibody was added into the 5% skim milk in TBST. Membranes were then developed using a Luminata Crescendo Western HRP substrate (Millipore). Primary antibodies used in this work are as follows: LC3 (L7543, Sigma), p62 (610832, BD Bioscience, Bedford, MA, USA), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (G9295, Sigma), α-tubulin (9099, Cell Signaling Technology, Danvers, MA, USA), CTSB (ab125067, Abcam, Cambridge, MA, USA), HA-tag (3724, Cell Signaling Technology), LAMP2 (ab125068, Abcam), phostensin (sc-365099, Santa Cruz Biotechnology, Santa Cruz, CA, USA), endophilin-3 (NBP1-79630, Novus, Littleton, CO, USA) and X2 box repressor (XBR, sc-25398, Santa Cruz Biotechnology). All primary antibodies were diluted at 1: 1000. The secondary HRP-conjugated anti-mouse (074-1806) or anti-rabbit antibodies (474-1506) were from KPL (Gaithersburg, Maryland, USA).

2.7. RNA expression analyses

Total RNA was isolated with RNAiso Plus (TAKARA) according to the manufacturer's instruction. For mRNA expression, total RNA was reverse transcribed to complementary DNA (cDNA) using the M-MLV Reverse Transcriptase (Promega, Madison, WI, USA), and then analyzed using real-time polymerase chain reaction (PCR) (SYBR Green) in