SHORT COMMUNICATION

Autophagy-Related Protein 7 Deficiency in Amyloid β ($A\beta$) Precursor Protein Transgenic Mice Decreases $A\beta$ in the Multivesicular Bodies and Induces $A\beta$ Accumulation in the Golgi

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Accepted for publication
October 14, 2014.

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Alzheimer disease (AD) is biochemically characterized by increased levels of amyloid β ($A\beta$) peptide, which aggregates into extracellular $A\beta$ plaques in AD brains. Before plaque formation, $A\beta$ accumulates intracellularly in both AD brains and in the brains of AD model mice, which may contribute to disease progression. Autophagy, which is impaired in AD, clears cellular protein aggregates and participates in $A\beta$ metabolism. In addition to a degradative role of autophagy in $A\beta$ metabolism we recently showed that $A\beta$ secretion is inhibited in mice lacking autophagy-related gene 7 (*Atg7*) in excitatory neurons in the mouse forebrain. This inhibition of $A\beta$ secretion leads to intracellular accumulation of $A\beta$. Here, we used fluorescence and immunoelectron microscopy to elucidate the subcellular localization of the intracellular $A\beta$ accumulation which accumulates in $A\beta$ precursor protein mice lacking *Atg7*. Autophagy deficiency causes accumulation of $p62^{+}$ aggregates, but these aggregates do not contain $A\beta$. However, knockdown of *Atg7* induced $A\beta$ accumulation in the Golgi and a concomitant reduction of $A\beta$ in the multivesicular bodies. This indicates that *Atg7* influences the transport of $A\beta$ possibly derived from Golgi to multivesicular bodies. (*Am J Pathol* 2015, 185: 305–313; <http://dx.doi.org/10.1016/j.ajpath.2014.10.011>)

Alzheimer disease (AD) is the most common form of dementia in the elderly. Biochemical hallmarks of AD include aggregation of intracellular Tau protein in neurofibrillary tangles and extracellular plaques that consist of amyloid β ($A\beta$) peptide. $A\beta$ is generated by sequential cleavage of $A\beta$ precursor protein (APP) by β - and γ -secretase. Mutations in *APP* or the in the genes encoding the enzymatic components of γ -secretase, *PSEN1* and *PSEN2*, lead to increased levels of $A\beta$, especially the aggregation-prone $A\beta_{42}$ and $A\beta_{43}$, and cause aggressive early-onset familial AD.^{1,2} Before extracellular $A\beta$ plaque formation, $A\beta$ accumulates intracellularly in AD brains and in the brains of AD model mice, which may contribute to disease progression.^{3,4} Transgene expression in mice of human ApoE4, which is a risk factor for AD, increases intraneuronal $A\beta$.⁵ Targeting $A\beta_{42}$ to the endoplasmic

reticulum (ER) induces neurodegeneration, suggesting that intraneuronal $A\beta$ may be toxic.⁶ However, the mechanistic link between $A\beta$ pathology and the extensive loss of synapses and neurons that occurs in AD remains largely unresolved.

AD brains also exhibit an accumulation of autophagosomes in the dystrophic neurites, indicating that autophagy is deregulated in AD.⁷ Macroautophagy (herein referred to as autophagy) is a cellular quality control system that degrades aberrant organelles and potentially toxic protein aggregates by delivering them to the lysosomes for degradation,

Supported by research grants from the Swedish Research Council, RIKEN Brain Science Institute, the Japanese Ministry of Education, Sports, Science and Technology, and the Japanese Ministry of Health, Labour and Welfare (P.N., M.S., K.H., M.T., K.S., T.S., and T.C.S.).

Disclosures: None declared.

reviewed by Nixon.⁸ The increased levels and abnormal distribution of lysosomal hydrolases in AD brains and in the brains of AD mouse models indicate that the end stage of autophagosomal clearance is impaired.^{9,10} Consistent with these reports, familial AD-associated mutations in *PSEN1* impair lysosomal proteolysis,¹¹ and pharmacologic inhibition of lysosomes in cultured neurons causes an AD-like accumulation of autophagosomes.¹²

Autophagosomes contain A β -generating enzymes and A β ,¹³ thus linking autophagy to A β metabolism.^{14,15} Rapamycin treatment induces autophagy that decreases intracellular A β and extracellular A β plaque load and improves cognition in AD model mice.^{16,17} Conversely, heterozygous deletion of autophagy-initiating Beclin1 increases intracellular and extracellular A β .^{18,19} Autophagy also influences the secretion of A β via a nondegradative route shown by genetic deletion of autophagy-related protein 7 (*Atg7*) in APP transgenic mice.²⁰ The inhibition of A β secretion leads to drastically decreased extracellular A β deposits.²⁰ Concomitantly, A β accumulates intracellularly which exacerbates the neurodegeneration caused by impaired autophagy and severely impairs the memory.²⁰ This finding adds A β to a growing number of proteins, the secretion of which is mediated by autophagy.²¹

Here, we investigated by immunofluorescence and immunoelectron microscopy (IEM) the secretory pathway of A β by tracing the intracellular A β that accumulates in *Atg7*-deficient APP transgenic mice. We found that genetic deletion of *Atg7* decreased A β in the multivesicular bodies (MVBs) and induced A β accumulation in the Golgi, suggesting that autophagy may mediate the trafficking of A β between the Golgi and the MVBs and that A β secretion to some extent occurs through the MVBs.

Materials and Methods

Animals

Atg7^{flox/flox} mice²² were provided by Keiji Tanaka (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan), and calcium/calmodulin-dependent protein kinase II (CaMKII)-*Cre* mice²³ were provided by Shigeyoshi Itoharu (RIKEN Brain Science Institute, Wako, Japan). APP23 mice have been described previously.²⁴ All mice were on C57BL/6 background. *Atg7^{flox/flox}* $\times$ APP mice and *Atg7^{flox/flox}*; CaMKII-*Cre* $\times$ APP mice used in the experiments were littermates. All animal experiments were conducted according to the guidelines of RIKEN Brain Science Institute.

Immunohistochemical Analysis

Paraffin-embedded brain sections were cut to 4 μ m and immunostained with antibodies against N1D-A β ,²⁵ A β 40 (no. 44348A; Invitrogen, Carlsbad, CA), APP-C-terminal fragment (CTF; no. A8717; Sigma-Aldrich, St. Louis, MO), p62 (no. GP62-C; Cell Signaling Technology Inc., Danvers, MA), vimentin (developed by Arturo Alvarez-Buylla and obtained from the Developmental Studies Hybridoma Bank

developed under the auspices of the National Institute of Child Health and Human Development and maintained by Department of Biology, The University of Iowa, Iowa City, IA), and cleaved caspase 3 (no. 9961; Cell Signaling Technology Inc.). For A β immunostaining, tissue sections were treated with 90% formic acid for 5 minutes. Quantification was performed with MetaMorph imaging software version 7.7.3.0 (Universal Imaging, Bedford Hills, NY).

Primary Neuron

Cortical/hippocampal neurons were prepared from mouse embryos (embryonic day 17 to 18) as previously described.²⁶ Embryos were separately genotyped. Vital cells (1.8×10^5) were plated in 24-well plates with poly-L-lysine-coated coverslips.

Immunofluorescence

Primary neurons were infected with Semliki Forest virus-APP for 24 hours, fixed for 10 minutes in cold 4% paraformaldehyde (PFA) and 0.1 mol/L phosphate buffer, washed with phosphate-buffered saline, blocked with 5% normal goat serum and 1% saponin, and incubated with antibodies N1D-A β ,²⁵ early endosome antigen 1 (no. 7659; Sigma-Aldrich), or microtubule-associated protein 2 (no. 5622; Millipore, Billerica, MA) overnight wash buffer and antibody dilutions contained 1% saponin.

Immunoblot Analysis

A β 40 (100 ng) and 10 μ g of delipidated brain homogenates were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with N1D-A β ,²⁵ A β 40 (no. 44348A; Invitrogen), 6E10 (no. SIG-39320; Covance Inc., Princeton, NJ), and APP-CTF (no. A8717; Sigma-Aldrich) antibodies.

IEM

For visualization of Golgi-localized A β , brains were perfused, fixed for 2 hours in 4% PFA and 0.25% glutaraldehyde, and cut into 200- μ m sections. Brain sections were incubated with 10%, 20%, and 30% sucrose; frozen; and thawed three times in liquid N₂. The brain sections were blocked in 10% bovine serum albumin, 10% normal goat serum, and 0.1% gelatin; incubated with N1D-A β ²⁵ antibody overnight; washed with phosphate-buffered saline that contained 0.1% gelatin; and incubated with 10 nm of nano-gold-conjugated secondary antibodies. After immunostaining, the sections were fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer for 1 hour at room temperature, washed with distilled H₂O, and intensified with the GoldEnhance EM (Nanoprobes, Yaphank, NY) to visualize the nano-gold labeling. After the intensification, the sections were osmicated with 1% OsO₄ and 1.5% potassium ferrocyanide in 0.1 mol/L phosphate buffer for 1 hour at room temperature,

Figure 1 *Atg7* deficiency induces intracellular A β accumulation. PFA-fixed and paraffin-embedded brain sections of 4- (A) and 9-month-old (B and C) *Atg7^{flox/flox} × APP* (grey bars) and *Atg7^{flox/flox}; CaMKII-Cre × APP* (black bars) littermates were immunostained with anti-N1D-A β antibody and anti-A β 40 antibody, as indicated. Perinuclear A β staining in CA1 pyramidal neurons was quantified. D: Specificities of A β antibodies. Synthetic A β 40 (100 ng) and 10 μ g delipidated brain homogenate from *Atg7^{flox/flox} × APP* mice were fractionated as indicated by gel-electrophoresis and analyzed by Western blot analysis. The membranes were probed with A β 40 and N1D-A β antibodies (lanes 1 and 3, respectively), stripped, and reprobed with A β /APP antibody (6E10; Covance) (lanes 2 and 4, respectively). E: Anti-N1D-A β and anti-A β 40 antibodies recognize aggregated A β in plaques of *Atg7^{flox/flox} × APP* and *Atg7^{flox/flox}; CaMKII-Cre × APP* mice. F: APP-CTF immunostaining of 9-month-old *Atg7^{flox/flox} × APP* mice (grey bars) and *Atg7^{flox/flox}; CaMKII-Cre × APP* (black bars) littermates. G: Quantitative Western blot analysis of dilapidated brain homogenate of *Atg7^{flox/flox} × APP* (grey bars) and *Atg7^{flox/flox}; CaMKII-Cre × APP* (black bars) mice, probed with antibodies as indicated. Data are expressed as means $\pm$ SEMs. $n = 7$ to 8 (A–C); $n = 5$ (E); $n = 4$ (F). * $P < 0.05$. Scale bars: 100 μ m (A–C and F); 500 μ m (E). A β , amyloid β ; APP, A β precursor protein; CA1, cornu ammonis 1; CaMKII, calcium/calmodulin-dependent protein kinase II; CTF, C-terminal fragment; PFA, paraformaldehyde.

dehydrated through a gradient series of ethanol concentrations, and embedded in epoxy resin (Epon 812; TAAB Laboratories Equipment Ltd., Berkshire, UK) by polymerization. Ultrathin sections (80 nm) were cut with an ultramicrotome (Ultracut UCT; Leica Microsystems, Wetzlar, Germany) and collected on copper grids. After counterstaining with uranyl acetate and lead citrate, the sections were examined by electron microscopy (Tecnai 12; FEI, Eindhoven, the Netherlands). For visualization of A β in MVBs, the procedure was performed as described previously.²⁷ Briefly, brains were perfused with 4% PFA, incubated overnight, and cut into 200- μ m thick sections. The

sections were embedded in LR white resin, cut into ultrathin sections, mounted on nickel grids, and incubated with anti-N1D-A β ²⁵ antibody, followed by colloidal gold-conjugated (10 nm) goat anti-rabbit IgG. The sections were viewed in transmission electron image mode (JEOL 1200EX; JEOL Ltd, Tokyo, Japan).

Statistical Analysis

Data were analyzed by Student's *t*-test and expressed as means $\pm$ SEMs.

Figure 2 Intracellular Aβ accumulation does not localize to p62⁺ aggregates. **A** and **B**: Coimmunostaining of p62 and aggresome marker vimentin with the use of paraffin-embedded brain sections of 9-month-old *Atg7^{fllox/flox}*, *Atg7^{fllox/flox}; CaMKII-Cre*, *Atg7^{fllox/flox} × APP*, and *Atg7^{fllox/flox}; CaMKII-Cre × APP* mice shows substantial colocalization of p62 and vimentin. **C** and **D**: p62 and Aβ coimmunostaining with paraffin-embedded brain sections of *Atg7^{fllox/flox} × APP* and *Atg7^{fllox/flox}; CaMKII-Cre × APP* littermates indicates no colocalization of Aβ with p62⁺ aggregates. Magnification of a single CA1 pyramidal neuron from *Atg7^{fllox/flox} × APP* and *Atg7^{fllox/flox}; CaMKII-Cre × APP* mice stained for p62 and Aβ (**D**). **E**: Autophagy deficiency induces cleavage of caspase 3 in *Atg7^{fllox/flox}; CaMKII-Cre* and *Atg7^{fllox/flox}; CaMKII-Cre × APP* mice. Scale bars: 50 μm (**A** and **C**); 20 μm (**B** and **E**); 5 μm (**D**). Original magnification: ×20 (**C**); ×40 (**D**). Aβ, amyloid β; APP, Aβ precursor protein; CA1, cornu ammonis 1; CaMKII, calcium/calmodulin-dependent protein kinase II.

Results

Aβ Accumulates Intracellularly in *Atg7*-Deficient APP Mice

We recently reported that autophagy influences the extracellular delivery of Aβ.²⁰ This was shown by using autophagy-deficient APP transgenic mice generated by crossbreeding *Atg7^{fllox/flox}* mice²² with *CaMKII-Cre* transgenic mice, which were further crossbred with APP23 mice. Lack of *Atg7* inhibited Aβ secretion which led to a drastically decreased Aβ plaque formation (Figure 1E).²⁰ In addition, immunohistochemical analysis found that Aβ accumulates intracellularly in cornu ammonis 1 (CA1) pyramidal neurons of 4-month-old autophagy-deficient *Atg7^{fllox/flox}; CaMKII-Cre × APP* mice compared with *Atg7^{fllox/flox} × APP* mice ($P < 0.05$) (Figure 1A). The accumulation further increases by age, as shown by

immunohistochemical experiments of 9-month-old mice with the use of two different Aβ-specific antibodies (N1D-Aβ²⁵ that recognizes the N1D structure of N-terminus of monomeric and aggregated Aβ and anti-Aβ40 antibody that recognizes the C-terminus of Aβ40 of monomeric and aggregated Aβ40) ($P < 0.05$) (Figure 1, B–E). In contrast, APP-CTF⁺ staining, APP levels, and APP-CTF levels were unchanged, although the APP-CTFβ/APP-CTFα ratio was slightly decreased in *Atg7^{fllox/flox}; CaMKII-Cre × APP* mice (Figure 1, F and G).

In agreement with previous studies,²⁸ autophagy deficiency in the CA1 neurons also leads to accumulation of protein aggregates that contain autophagic adaptor protein p62 (Figure 2A). Coimmunostaining experiments found that the p62⁺ aggregates contain vimentin, suggesting that these structures are aggresomes (Figure 2, A and B). In contrast, the p62⁺ aggregates did not colocalize with Aβ, indicating that the site of intracellular Aβ buildup is not

Figure 3 *Atg7* deficiency induces A β accumulation in the Golgi. **A:** Immunoelectron micrographs of brain tissues with genotypes as indicated with the use of 4% PFA and 0.25% GA for tissue fixation shows fibrillary aggregates in *Atg7^{fllox/fllox}*; CaMKII-Cre x APP mice. **B:** Immunoelectron micrograph overviews of brain sections from 9-month-old APP knockout, *Atg7^{fllox/fllox}* x APP, and *Atg7^{fllox/fllox}*; CaMKII-Cre x APP mice as indicated. **Arrowheads** indicate A β immunogold labeling in the Golgi and the MVBs. **C:** Higher amplification of immunoelectron micrographs of the Golgi of brain sections from APP knockout (white bar), *Atg7^{fllox/fllox}* x APP (gray bar), and *Atg7^{fllox/fllox}*; CaMKII-Cre x APP (black bar) mouse as indicated. The number of gold-labeled particles/Golgi was quantified. Data are expressed as means $\pm$ SEM. $n > 40$ gold-labeled particles/Golgi (**C**). $**P < 0.01$; three individual mice per genotype were investigated except for one APP knockout mouse. Scale bars: 1 μ m (**A**); 200 nm (**B**); 100 nm (**C**). A β , amyloid β ; Agg, aggregate; APP, A β precursor protein; CaMKII, calcium/calmodulin-dependent protein kinase II; ER, endoplasmic reticulum; GA, glutaraldehyde; Go, Golgi; KO, knockout; Mito, mitochondria; MVB, multivesicular body; Nuc, nucleus; PFA, paraformaldehyde.

associated with these intracellular aggregates (Figure 2, C and D). Furthermore, cleavage of caspase 3 is detected in *Atg7^{fllox/fllox}*;CaMKII-Cre mice, which is exacerbated in the *Atg7^{fllox/fllox}*;CaMKII-Cre x APP mice (Figure 2E). The presence of cleaved caspase 3 suggests an onset of apoptotic processes, in agreement with previous data which indicated a significant neurodegeneration in 15-month-old *Atg7^{fllox/fllox}*;CaMKII-Cre and *Atg7^{fllox/fllox}*;CaMKII-Cre x APP mice.²⁰

A β Accumulates in the Golgi on Genetic Deletion of *Atg7*

To get an insight into how the A β secretory pathway is affected in the autophagy-deficient *Atg7^{fllox/fllox}*;CaMKII-Cre x APP mice, we investigated the subcellular localization of the intracellular A β accumulation that occurs in

the brains of 9-month-old mice by IEM. To establish conditions for IEM that preserve the ultrastructure of the tissue but partially maintain the antigenicity of A β , the effect of different concentrations of glutaraldehyde and different time of tissue fixation was investigated. The ultrastructure of the brain tissue was well preserved in a fixation method that was based on 4% PFA and 0.25% glutaraldehyde (see *Materials and Methods*), which allowed structural identification of the cellular organelles, including the aggregates in the perinuclear region (Figure 3A). With the use of this fixation protocol, we localized A β by immunolabeling brain tissue from an APP knockout mouse and *Atg7^{fllox/fllox}* x APP and *Atg7^{fllox/fllox}*;CaMKII-Cre x APP littermates with A β antibodies and gold-labeled secondary antibodies and analyzed the samples by IEM. Even though the gold labeling was sparse, signal was detected in the Golgi and

the MVBs in the pyramidal cells in CA1 (Figure 3B). By studying numerous CA1 pyramidal cells, a significant increase of gold-labeled A β in the Golgi was observed in the autophagy-deficient CA1 neurons of *Atg7^{fl/fl}/fl^{ox}*; *CaMKII-Cre* $\times$ APP mice compared with *Atg7^{fl/fl}/fl^{ox}* $\times$ APP mice ($n > 40$; $P < 0.005$) (Figure 3C). Immunogold-labeled A β was found at the *cis*- and *trans*-face of the Golgi and was associated with both the membrane of formed vesicles in the late Golgi apparatus and the cisternae. Consistent with the immunofluorescence data (Figure 2, C and D), no A β was detected in the fibrillary aggregates in the *Atg7^{fl/fl}/fl^{ox}*; *CaMKII-Cre* $\times$ APP mice (Figure 3A).

Atg7 Deficiency Leads to Decreased A β in the MVBs

In an attempt to increase the antigenicity of A β , and thereby the IEM signal, we applied a milder fixation protocol that was based on only 4% PFA. Although this protocol did not fully preserve the ultrastructure of the ER and the Golgi, as in the glutaraldehyde-based fixation method, some structures within neurites were indeed preserved, including the MVBs (Figure 4A). Previous reports have found that the MVBs contain significant amounts of A β .²⁹ Therefore, we used this fixation method and gold-labeled A β in brain tissue from *Atg7^{fl/fl}/fl^{ox}* $\times$ APP and *Atg7^{fl/fl}/fl^{ox}*; *CaMKII-Cre* $\times$ APP littermates and subjected the samples to IEM. In agreement with earlier studies, gold-labeled A β decorated the membranes of internalized vesicles in the MVBs in neurites of *Atg7^{fl/fl}/fl^{ox}* $\times$ APP mice (Figure 4, A and B). In contrast, the A β ⁺ signal in the MVBs in the neurites of the autophagy-deficient *Atg7^{fl/fl}/fl^{ox}*; *CaMKII-Cre* $\times$ APP mice was significantly decreased ($P < 0.05$) (Figure 4, A and B).

To further study the effect of *Atg7* deficiency on A β localization, primary neurons derived from *Atg7^{fl/fl}/fl^{ox}* and *Atg7^{fl/fl}/fl^{ox}*; *Nes-Cre* mice were infected with Semliki Forest virus that expressed wild-type APP and subsequently stained for A β . Interestingly, *Atg7* deficiency induced accumulation of A β in the perinuclear region, whereas less A β localized to the neurites (Figure 4, C and D). The punctuate A β staining in the neurites of autophagy-competent neurons was identified as endosomes, the A β content of which was decreased in the *Atg7*-deficient neurons (Figure 4E).

Figure 4 Lack of *Atg7* decreases A β in the MVBs of *Atg7^{fl/fl}/fl^{ox}*; *CaMKII-Cre* $\times$ APP mice. **A:** Immunoelectron micrograph overviews of neurons from 9-month-old mice with genotypes as indicated by using 4% PFA for tissue fixation. **B:** Higher magnification of micrographs show immunogold-labeled A β in the MVBs in neurites of *Atg7^{fl/fl}/fl^{ox}* $\times$ APP (gray bar) and *Atg7^{fl/fl}/fl^{ox}*; *CaMKII-Cre* $\times$ APP (black bar) mice. The A β signal in the MVBs was quantified. **C–E:** Immunofluorescence experiments using primary neurons derived from mice with genotypes as indicated and infected with Semliki Forest virus-APP. Primary neurons were stained for A β (C), A β and microtubule-associated protein 2 (D), and A β and early endosome antigen 1 (E). Data are expressed as means $\pm$ SEM. $n = 3$ (B). $*P < 0.05$. Scale bars: 1 μ m (A); 250 nm (B); 50 nm (C and D). A β , amyloid β ; APP, A β precursor protein; CaMKII, calcium/calmodulin-dependent protein kinase II; MVB, multivesicular body; PFA, paraformaldehyde.

Together, these data indicate that Atg7 influences the sorting of A β in the cell and that autophagosomes may be involved in the transport of A β between the Golgi and the MVBs.

Discussion

The accumulation of autophagosomes in the dystrophic neurites of AD brains shows that autophagy is dysregulated in AD, although whether it is because of increased initiation of autophagy or decreased lysosomal clearance remains to be established. In addition, a number of studies have associated autophagy with two of the biochemical hallmarks of AD: A β and Tau.³⁰ The link between A β and autophagy is supported by the direct observation that the autophagosomes contain A β and that manipulation of autophagy by genetic or pharmacologic means affects the A β pathology of AD model mice. The role of autophagy in A β metabolism was interpreted to be degradation of A β within the autophagosomal-lysosomal network. However, we recently extended the knowledge about the role of autophagy in A β metabolism by showing that A β secretion, and hence A β plaque formation, depends on autophagy.²⁰ Therefore, autophagy may target A β for either degradation or secretion.

A β is produced from APP in two major pathways; APP translocated from the ER is processed within the Golgi from where secretory vesicles transport A β to the plasma membrane where A β is secreted or unprocessed APP located at the plasma membrane is endocytosed and A β is cleaved in late endosomes/MVBs.³¹ Here, we investigated how A β transport and secretion were affected on genetic deletion of Atg7 by using IEM to determine the subcellular localization of intracellular A β that accumulates in the autophagy-deficient Atg7^{fllox/fllox};CaMKII-Cre $\times$ APP mice (Figures 1 and 2). Interestingly, we found that Atg7 deficiency leads to A β accumulation in the Golgi, whereas the levels of A β in the MVBs are significantly decreased (Figures 3 and 4). Whether this redistribution of A β is caused by a lack of autophagosomes per se or is because of the absence of Atg7 protein, and therefore independent of autophagosomes, remains to be elucidated.

A role for autophagy in protein secretion has recently emerged (reviewed in Deretic et al³² and Zhang and Schekman³³). Autophagy regulates protein secretion by intersecting with conventional constitutive, unconventional, and regulated secretory pathways. Autophagy-mediated conventional secretion of interleukin 6 and 8, for example, is initiated at the TOR (target of rapamycin)-autophagy spatial coupling compartment. This compartment is closely located to the Golgi and is sensitive to Brefeldin A, which indicates a close connection of this secretory pathway to the classic constitutive ER-Golgi-plasma membrane secretory pathway.³⁴ Autophagy also mediates the unconventional secretion of a number of cytoplasmic proteins that lack a transport signal peptide, including acetyl-coenzyme binding protein 1.^{35,36}

Unconventional protein secretion is initiated from a recently identified structure termed omegasome. Although the current knowledge about the omegasome is limited, it is described as a membranous structure found on the ER, and its function depends on the Golgi reassembly and stacking protein. The A β accumulation in the Golgi induced by Atg7 deficiency indicates that the Golgi or Golgi-associated structures are involved in A β secretion influenced by Atg7. Indeed, it was found that A β is generated in the Golgi after retromer transport of endocytosed APP, a process that depends on factors involved in the formation of MVBs.³⁷ Interestingly, mutations in genes involved in retromer transport, including Rab7A, have been identified in some AD patients.³⁸ This indicates that autophagy-mediated transport between the Golgi and the endosomes/MVBs may play a role in A β secretion. In this context, it is worth noting that the membrane source for the autophagosomes may include the ER, the Golgi, mitochondria, and the plasma membrane (reviewed in Rubinstein et al³⁹) and that APP, which is a membrane-bound protein, could potentially be recruited along with the membrane on autophagosome formation.

Unconventional secretion of acetyl-coenzyme binding protein 1 additionally depends on factors important for the generation of the MVBs, which indicates that the amphisome is involved in the secretion of acetyl-coenzyme binding protein 1. We found that the A β content in the MVBs is decreased on genetic deletion of Atg7, suggesting that A β is delivered to the MVBs by autophagy and may be exocytosed via amphisomes. Indeed, A β is released, at least to some extent, to the extracellular space in exosomes.⁴⁰ However, autophagy also participates in regulated secretion from lysosomes, and A β secretion is decreased as secretion from lysosomes is decreased.⁴¹ Further research is needed to elucidate the contribution of each of these secretory pathways to total A β secretion and in which pathway autophagy is involved. Interestingly, recent research has established that Tau protein is secreted, at least partially, in exosomes.⁴² Moreover, Tau protein is metabolized by autophagy³⁰; therefore, a role for autophagy in Tau secretion cannot be excluded.

Intracellular A β accumulation in the late endosomes, which are closely related to the MVBs, has been found both in AD brain and in the brains of AD model mice, but whether this is directly linked to increased autophagy remains to be clarified. Nevertheless, increasing evidence indicates that intracellular A β accumulation is toxic, a conclusion that is supported by our previous work showing that A β amyloidosis (including intracellular A β) exacerbates the neurodegeneration associated with autophagy deficiency.²⁰ Therefore, understanding the A β secretory pathway and its interplay with autophagy is of uttermost importance.

Acknowledgments

We thank Keiji Tanaka and Shigeyoshi Itohara for generously providing Atg7^{fllox/fllox} and CaMKII-Cre mice,

respectively, Takashi Suematsu for help with electron microscopy measurements and the following members of the PNS laboratory: Jiro Takano, Satoshi Tsubuki, Ko Sato, Kenichi Nagata, Naomasa Kakiya, Shoko Hashimoto, Hayato Isshiki, Kaori Tsukakoshi, Yukio Matsuba, Emi Hosoki, Ryo Fujioka, Naomi Yamazaki, Yuya Tomita, and Yukiko Nagai.

References

- Haass C, Kaether C, Thinakaran G, Sisodia S: Trafficking and Proteolytic Processing of APP. *Cold Spring Harb Perspect Med* 2012, 2: a006270
- Saito T, Suemoto T, Brouwers N, Slegers K, Funamoto S, Mihira N, Matsuba Y, Yamada K, Nilsson P, Takano J, Nishimura M, Iwata N, Van Broeckhoven C, Ihara Y, Saido TC: Potent amyloidogenicity and pathogenicity of A β 43. *Nat Neurosci* 2011, 14:1023–1032
- LaFerla FM, Green KN, Oddo S: Intracellular A β in Alzheimer's disease. *Nat Rev Neurosci* 2007, 8:499–509
- Wirths O, Bayer TA: Intraneuronal A β accumulation and neurodegeneration: lessons from transgenic models. *Life Sci* 2012, 91: 1148–1152
- Zhao W, Dumanis SB, Tamboli IY, Rodriguez GA, Jo LaDu M, Moussa CE, William Rebeck G: Human APOE genotype affects intraneuronal A β 1–42 accumulation in a lentiviral gene transfer model. *Hum Mol Genet* 2013, 23:1365–1375
- Abramowski D, Rabe S, Upadhyaya AR, Reichwald J, Danner S, Staab D, Capetillo-Zarate E, Yamaguchi H, Saido TC, Wiederhold KH, Thal DR, Staufenbiel M: Transgenic expression of intraneuronal A β 42 but not A β 40 leads to cellular A β lesions, degeneration, and functional impairment without typical Alzheimer's disease pathology. *J Neurosci* 2012, 32:1273–1283
- Nixon RA, Wegiel J, Kumar A, Yu WH, Peterhoff C, Cataldo A, Cuervo AM: Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study. *J Neuropathol Exp Neurol* 2005, 64:113–122
- Nixon RA: The role of autophagy in neurodegenerative disease. *Nat Med* 2013, 19:983–997
- Cataldo AM, Paskevich PA, Kominami E, Nixon RA: Lysosomal hydrolases of different classes are abnormally distributed in brains of patients with Alzheimer disease. *Proc Natl Acad Sci U S A* 1991, 88: 10998–11002
- Yang DS, Stavrides P, Mohan PS, Kaushik S, Kumar A, Ohno M, Schmidt SD, Wesson D, Bandyopadhyay U, Jiang Y, Pawlik M, Peterhoff CM, Yang AJ, Wilson DA, St George-Hyslop P, Westaway D, Mathews PM, Levy E, Cuervo AM, Nixon RA: Reversal of autophagy dysfunction in the TgCRND8 mouse model of Alzheimer's disease ameliorates amyloid pathologies and memory deficits. *Brain* 2011, 134:258–277
- Lee JH, Yu WH, Kumar A, Lee S, Mohan PS, Peterhoff CM, Wolfe DM, Martinez-Vicente M, Massey AC, Sovak G, Uchiyama Y, Westaway D, Cuervo AM, Nixon RA: Lysosomal proteolysis and autophagy require presenilin 1 and are disrupted by Alzheimer-related PS1 mutations. *Cell* 2010, 141:1146–1158
- Boland B, Kumar A, Lee S, Platt FM, Wegiel J, Yu WH, Nixon RA: Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease. *J Neurosci* 2008, 28:6926–6937
- Yu WH, Cuervo AM, Kumar A, Peterhoff CM, Schmidt SD, Lee JH, Mohan PS, Mercken M, Farmery MR, Tjernberg LO, Jiang Y, Duff K, Uchiyama Y, Näslund J, Mathews PM, Cataldo AM, Nixon RA: Macroautophagy—a novel β -amyloid peptide-generating pathway activated in Alzheimer's disease. *J Cell Biol* 2005, 171: 87–98
- Boland B, Smith DA, Mooney D, Jung SS, Walsh DM, Platt FM: Macroautophagy is not directly involved in the metabolism of amyloid precursor protein. *J Biol Chem* 2010, 285:37415–37426
- Jaeger PA, Pickford F, Sun CH, Lucin KM, Masliah E, Wyss-Coray T: Regulation of amyloid precursor protein processing by the Beclin 1 complex. *PLoS One* 2010, 5:e11102
- Caccamo A, Majumder S, Richardson A, Strong R, Oddo S: Molecular interplay between mammalian target of rapamycin (mTOR), amyloid- β , and Tau: effects on cognitive impairments. *J Biol Chem* 2010, 285: 13107–13120
- Majumder S, Richardson A, Strong R, Oddo S: Inducing autophagy by rapamycin before, but not after, the formation of plaques and tangles ameliorates cognitive deficits. *PLoS One* 2011, 6:e25416
- Pickford F, Masliah E, Britschgi M, Lucin K, Narasimhan R, Jaeger PA, Small S, Spencer B, Rockenstein E, Levine B, Wyss-Coray T: The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid β accumulation in mice. *J Clin Invest* 2008, 118:2190–2199
- Salminen A, Kaamiranta K, Kauppinen A, Ojala J, Haapasalo A, Soininen H, Hiltunen M: Impaired autophagy and APP processing in Alzheimer's disease: the potential role of Beclin 1 interactome. *Prog Neurobiol* 2013, 106–107:33–54
- Nilsson P, Loganathan K, Sekiguchi M, Matsuba Y, Hui K, Tsubuki S, Tanaka M, Iwata N, Saito T, Saido TC: A β secretion and plaque formation depend on autophagy. *Cell Rep* 2013, 5:61–69
- Nilsson P, Saido TC: Dual roles for autophagy: degradation and secretion of Alzheimer's disease A β peptide. *Bioessays* 2014, 36: 570–578
- Komatsu M, Waguri S, Ueno T, Iwata J, Murata S, Tanida I, Ezaki J, Mizushima N, Ohsumi Y, Uchiyama Y, Kominami E, Tanaka K, Chiba T: Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J Cell Biol* 2005, 169:425–434
- Tsien JZ, Chen DF, Gerber D, Tom C, Mercer EH, Anderson DJ, Mayford M, Kandel ER, Tonegawa S: Subregion- and cell type-restricted gene knockout in mouse brain. *Cell* 1996, 87: 1317–1326
- Sturchler-Pierrat C, Abramowski D, Duke M, Wiederhold KH, Mistl C, Rothacher S, Ledermann B, Bürki K, Frey P, Paganetti PA, Waridel C, Calhoun ME, Jucker M, Probst A, Staufenbiel M, Sommer B: Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology. *Proc Natl Acad Sci U S A* 1997, 94:13287–13292
- Saido TC, Yokota M, Maruyama K, Yamao-Harigaya W, Tani E, Ihara Y, Kawashima S: Spatial resolution of the primary beta-amyloidogenic process induced in postischemic hippocampus. *J Biol Chem* 1994, 269:15253–15257
- Hama E, Shirotani K, Masumoto H, Sekine-Aizawa Y, Aizawa H, Saido TC: Clearance of extracellular and cell-associated amyloid β peptide through viral expression of neprilysin in primary neurons. *J Biochem* 2001, 130:721–726
- Goto S, Kawakatsu M, Izumi S, Urata Y, Kageyama K, Ihara Y, Koji T, Kondo T: Glutathione S-transferase π localizes in mitochondria and protects against oxidative stress. *Free Radic Biol Med* 2009, 46:1392–1403
- Inoue K, Rispoli J, Kaphzan H, Klann E, Chen EI, Kim J, Komatsu M, Abeliovich A: Macroautophagy deficiency mediates age-dependent neurodegeneration through a phospho-tau pathway. *Mol Neurodegener* 2012, 7:48–60
- Takahashi RH, Milner TA, Li F, Nam EE, Edgar MA, Yamaguchi H, Beal MF, Xu H, Greengard P, Gouras GK: Intraneuronal Alzheimer a β 42 accumulates in multivesicular bodies and is associated with synaptic pathology. *Am J Pathol* 2002, 161:1869–1879
- Lee MJ, Lee JH, Rubinstein DC: Tau degradation: the ubiquitin–proteasome system versus the autophagy-lysosome system. *Prog Neurobiol* 2013, 105:49–59
- O'Brien RJ, Wong PC: Amyloid precursor protein processing and Alzheimer's disease. *Annu Rev Neurosci* 2011, 34:185–204

32. Deretic V, Jiang S, Dupont N: Autophagy intersections with conventional and unconventional secretion in tissue development, remodeling and inflammation. *Trends Cell Biol* 2012, 22: 397–406
33. Zhang M, Schekman R: Cell biology. Unconventional secretion, unconventional solutions. *Science* 2013, 340:559–561
34. Narita M, Young AR, Arakawa S, Samarajiwa SA, Nakashima T, Yoshida S, Hong S, Berry LS, Reichelt S, Ferreira M, Tavaré S, Inoki K, Shimizu S, Narita M: Spatial coupling of mTOR and autophagy augments secretory phenotypes. *Science* 2011, 332:966–970
35. Duran JM, Anjard C, Stefan C, Loomis WF, Malhotra V: Unconventional secretion of Acb1 is mediated by autophagosomes. *J Cell Biol* 2010, 188:527–536
36. Manjithaya R, Anjard C, Loomis WF, Subramani S: Unconventional secretion of *Pichia pastoris* Acb1 is dependent on GRASP protein, peroxisomal functions, and autophagosome formation. *J Cell Biol* 2010, 188:537–546
37. Choy RW, Cheng Z, Schekman R: Amyloid precursor protein (APP) traffics from the cell surface via endosomes for amyloid β (A β) production in the trans-Golgi network. *Proc Natl Acad Sci U S A* 2012, 109:E2077–E2082
38. Vardarajan BN, Bruesegem SY, Harbour ME, Inzelberg R, Friedland R, St George-Hyslop PS, Seaman MN, Farrer LA: Identification of Alzheimer disease-associated variants in genes that regulate retromer function. *Neurobiol Aging* 2012, 33:2231.e15–2231.e30
39. Rubinsztein DC, Shpilka T, Elazar Z: Mechanisms of autophagosome biogenesis. *Curr Biol* 2012, 22:R29–R34
40. Rajendran L, Honsho M, Zahn TR, Keller P, Geiger KD, Verkade P, Simons K: Alzheimer's disease β -amyloid peptides are released in association with exosomes. *Proc Natl Acad Sci U S A* 2006, 103: 11172–11177
41. Annunziata I, Patterson A, Helton D, Hu H, Moshiaich S, Gomero E, Nixon R, d'Azzo A: Lysosomal NEU1 deficiency affects amyloid precursor protein levels and amyloid- β secretion via deregulated lysosomal exocytosis. *Nat Commun* 2013, 4:2734
42. Gendreau KL, Hall GF: Tangles, toxicity, and tau secretion in AD – new approaches to a vexing problem. *Front Neurol* 2013, 4:160