

The Ubiquitin–Proteasome System and the Autophagic–Lysosomal System in Alzheimer Disease

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As neurons age, their survival depends on eliminating a growing burden of damaged, potentially toxic proteins and organelles—a capability that declines owing to aging and disease factors. Here, we review the two proteolytic systems principally responsible for protein quality control in neurons and their important contributions to Alzheimer disease pathogenesis. In the first section, the discovery of paired helical filament ubiquitination is described as a backdrop for discussing the importance of the ubiquitin–proteasome system in Alzheimer disease. In the second section, we review the prominent involvement of the lysosomal system beginning with pathological endosomal–lysosomal activation and signaling at the very earliest stages of Alzheimer disease followed by the progressive failure of autophagy. These abnormalities, which result in part from Alzheimer-related genes acting directly on these lysosomal pathways, contribute to the development of each of the Alzheimer neuropathological hallmarks and represent a promising therapeutic target.

Cellular aging, the *sine qua non* for Alzheimer disease development, is associated with cumulative oxidative damage to proteins and membranes, translational errors leading to the synthesis of defective proteins, and various genetic and environmental insults to organelles and proteins (Terman 2001; Roy et al. 2002; Sohal et al. 2002; Troen 2003; Levine et al. 2004). In some aging-related neurodegenerative

diseases, mutations of the pathogenic gene(s) may also cause anomalous conformations of the encoded protein or its metabolites, leading to increased proteolytic resistance and accumulation of these potentially toxic proteins (Soto 2003). A neuron's ability to function in the face of these mounting aging- and disease-related insults is determined in significant part by the efficiency with which it can eliminate

Editors: Dennis J. Selkoe, Eckhard Mandelkow, and David M. Holtzman
Additional Perspectives on The Biology of Alzheimer Disease available at www.perspectivesinmedicine.org

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Cite this article as *Cold Spring Harb Perspect Med* 2012;2:a006361

Y. Ihara et al.

this burden of damaged cellular constituents. An array of proteases regulates cell function, but two proteolytic systems are mainly responsible for the turnover of proteins and organelles—the proteasome and lysosomal systems (Grune et al. 2001; Berke et al. 2003; Goldberg 2003; Levine et al. 2004). The proteasome selectively degrades normal proteins (mainly those with short half-lives) and abnormal proteins, which are earmarked for elimination by a process involving their conjugation to ubiquitin (Ub; Goldberg 2003). The lysosomal system, and specifically the autophagic pathway, is the principal mechanism for degrading proteins with long half-lives and is the only system in cells for degrading organelles and large protein aggregates or inclusions. A second route to lysosomes, the endocytic pathway, delivers extracellular material and plasma membrane constituents to lysosomes under the direction of specific targeting signals (Nixon 2004).

Not surprisingly, protein quality control and proteostasis—the appropriate balance of protein synthesis, folding, and turnover in the cell—involve interdependent regulation of these two proteolytic systems. This interdependence is best exemplified by the regulatory influences of the protein ubiquitin. It has recently become appreciated that ubiquitination of proteins by covalent modification tags them for elimination not only through the proteasome (the ubiquitin–proteasome system or UPS) but also through the lysosomal system. Endocytosed receptors are targeted for lysosomal degradation, in part, by ubiquitination in late endosomes/multivesicular bodies (MVBs). Recently, protein aggregates and certain organelles have been shown to be tagged with ubiquitination for selective removal by autophagy (Narendra et al. 2009; Dikic et al. 2010; Youle et al. 2011), a degradative process previously believed to be only nonselective. In selective autophagy, an adaptor molecule with a ubiquitin-binding domain engages the ubiquitinated structure and couples it to the pre-autophagosomal isolation membrane for subsequent sequestration. The exact types of ubiquitin motifs recognized by the UPS and

autophagy may differ and the degree to which ubiquitination drives autophagic protein turnover relative to that by proteasomes is still unclear. Nevertheless, the role of ubiquitin as a tag for targeting substrates for elimination is more universal than earlier imagined, implying that changes in ubiquitin balance may be one way that disease-related perturbations of one proteolytic system are likely to influence the other.

Interdependence of the proteasome and lysosomal system is also suggested by observations that, when proteasome activity is inhibited, proteins accumulate that become substrates for autophagy (Fortun et al. 2003). Inhibiting autophagy by genetically deleting components of the sequestration machinery causes ubiquitinated protein aggregates to appear in neurons, reflecting additional negative effects on the UPS (Korolchuk et al. 2009a,b). Besides ubiquitin, proteins identified to play regulatory roles in the UPS and autophagy are being increasingly identified (Zhao et al. 2007). For example, p62, an adaptor protein for autophagy, also influences proteasomal degradation, whereas VCP/p97 acting through p62 and ubiquitin regulates both the proteasome-dependent endoplasmic reticulum-associated degradation (ERAD) pathway and aspects of autophagosome maturation (Tresse et al. 2010). The E3 ligase Parkin, a protein implicated in Parkinson's disease, creates an autophagy signal on mitochondria and also tags proteins elsewhere for proteasomal degradation (Yoshii et al. 2011). Interestingly, proteasomal subunits may be degraded by lysosomes (Cuervo et al. 1995), hinting at an additional level of cross talk between these proteolytic systems.

Proteolysis has been an active area of Alzheimer disease research over the past two decades mainly in relation to the processing of specific proteins, like the β -amyloid precursor protein. A broader appreciation is emerging, however, of the roles of proteolytic systems in other crucial aspects of Alzheimer disease (AD) pathogenesis related to protein clearance, neural plasticity, and neurodegenerative mechanisms. In this article, we focus on the involvement of the UPS and the lysosomal system (endosomal–lysosomal pathway and autophagy) in AD,

their contributions to disease development and progression, and the possibilities for targeting these systems in the design of innovative therapies for AD.

PART I: THE UBIQUITIN–PROTEASOME SYSTEM IN AD

Discovery of Ubiquitin in Paired Helical Filaments: A Personal Retrospective by Yasuo Ihara

Polyclonal antibodies to the classical paired helical filaments (PHFs) found in the neurofibrillary tangles and dystrophic neurites of AD were first raised in about 1982, allowing exploration of the component(s) of PHFs using immunochemical approaches (Ihara et al. 1983). This strategy was useful because PHFs were resistant to conventional protease digestion, and it was difficult to obtain reproducible and distinct limited-digest protein fragments of PHFs for sequencing. Sodium dodecyl sulfate (SDS)-insoluble material was extracted from typical AD brains, and PHFs were partially purified from the SDS-insoluble material by differential centrifugation and sucrose density gradient centrifugation (Ihara et al. 1983). The PHFs obtained by these procedures were not yet sufficiently pure, as shown later by sequencing of fragments of PHF proteins (Kondo et al. 1988). The polyclonal PHF antisera that were initially raised also reacted with lipofuscin-like granules and other SDS-insoluble materials in the PHF-rich immunogen. A major frustration with the antisera was that, although they intensely stained neurofibrillary tangles (NFTs), dystrophic neurites, and neuropil threads in AD brain sections, they did not label a distinct band(s) on immunoblots of AD brain homogenates, instead giving peculiar smears running from very high to low molecular mass (Ihara et al. 1983). These immunoreactive smears were highly characteristic of AD brain homogenates and never seen in control brain homogenates. Thus, the indirect immunochemical approach to identify the PHF component proteins seemed unsuccessful at first. However, it

succeeded a few years later when fetal or neonatal brain homogenates were probed with anti-PHF antisera: A somewhat diffuse band around 50 kDa was intensely labeled in immunoblots of fetal brain homogenates. This protein reactive with the initial anti-PHF sera was soon identified as tau, a microtubule-associated protein (MAP), based on its molecular weight, isoform change during development, microtubule-binding activity, and heat stability (Kosik et al. 1986; Nukina and Ihara 1986; see also Brion et al. 1985; Grundke-Iqbal et al. 1986; Wood et al. 1986; discovery of tau in PHF is reviewed in Mandelkow and Mandelkow 2011).

In contrast to our initial PHF antisera, we assumed that a monoclonal antibody that recognizes a highly defined epitope on PHF would not give rise to the smearing pattern on immunoblots that greatly puzzled us. In 1985, we became aware of a hybrid system (Pike et al. 1982) for raising monoclonal antibodies to glycolipids. Lewis rats responded well to immunizations with partially purified PHF preparations, yielding high titers of antisera to PHFs. These rats provided splenocytes for fusion with mouse myeloma cells. We (Hiroshi Mori and Yasuo Ihara) employed a two-step screening procedure to identify monoclonals in the resultant hybridoma supernatants: first by enzyme-linked immunosorbent assay (ELISA) using plates coated with partially purified PHFs, and second by immunostaining of NFTs that were prepared from AD brains under nondenaturing conditions. Only two confirmed and stable cell lines emerged, the antibodies from which had very similar specificities on the blots: Besides the characteristic “PHF smear,” they also labeled a very small protein of ~8 kDa. Hiroshi Mori named these monoclonal antibodies DF (Dementia Filament) 1 and 2, of which the latter (DF2) was used for subsequent characterization (Mori et al. 1987).

In contrast to our PHF antisera, DF2 produced immunoreactive smears even in control brain homogenates (presumably representing Ub-conjugated proteins). In addition, DF2 gave a distinct band at 8 kDa on blots of aged human brain homogenates, whereas the PHF

Y. Ihara et al.

antisera had not given distinct bands. We were puzzled as to why the two antibodies labeled different bands, but hypothesized that they recognized different components of PHFs. Optical diffraction analyses carried out by Wischik et al. (1985) had suggested that the core of the PHF may be composed of a subunit around ~100 kDa. We reasoned that one protein might make up the core framework of PHFs, whereas another might occur on a peripheral portion.

In AD cortical sections, we observed that NFTs and dystrophic neurites (Fig. 1) and, unexpectedly, granulovacuolar changes (Fig. 1, inset) were intensely immunolabeled by the DF2 monoclonal. When mild fixation conditions were used, innumerable neuropil threads were also detected. (One peculiar characteristic was an apparent “background” staining of AD brain sections, whereas widely used Ub polyclonal antibodies [Haas and Bright 1985] gave almost no background staining. This discrepancy was resolved in 1996: DF2 is specific for the conjugated form of Ub, whereas the Ub polyclonal antibodies are specific for free Ub [Morimoto et al. 1996].)

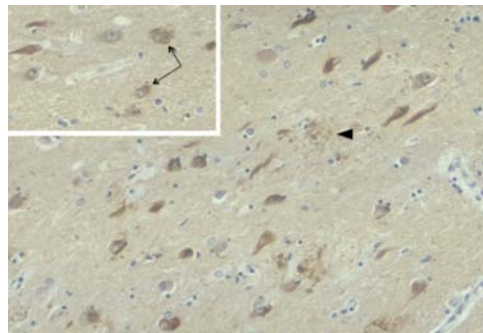


Figure 1. DF2 immunostaining of a tissue section from Alzheimer disease (AD) hippocampus. Neurofibrillary tangles and dystrophic neurites (arrowhead) are intensely labeled, whereas the neuropil (background) is uniformly stained, simulating non-specific background staining. However, this was found to be true staining of ubiquitin-conjugated proteins in the neuropil (Morimoto et al. 1996). (*Inset*) Some neurons in CA1 undergo granulovacuolar changes (arrows) and are also intensely stained with DF2.

What was the small protein at 8 kDa that was strongly labeled by DF2? Purifying the protein was not difficult with high-performance liquid chromatography (HPLC), which was beginning to be used for protein fractionation. The sequence of the DF2-reactive protein up to approximately 30 residues exactly corresponded to that of ubiquitin (Mori et al. 1987). This was probably the first description of Ub protein modification in the field of neuropathology. Two seminal papers describing the significance of protein ubiquitination had recently been published by the Varshavsky laboratory (Ciechanover et al. 1984; Finley et al. 1984). Despite reading these papers, we could not understand why Ub is present on PHFs. Our understanding was that Ub is a tag for protein degradation: A Ub-tagged protein should be recognized and degraded by the proteasome, an unusually large cytoplasmic protease. However, Ub was found to have other functional roles; for example, a proportion of histone (H)2B is ubiquitinated in a way that has a role in the transcription.

Ubiquitin appeared to represent a novel constituent of PHFs; DF2 strongly stained SDS-stripped NFTs, suggesting that Ub was an integral component. Nevertheless, we felt that the immunochemical identification of the component was not sufficient to fully confirm the presence of the molecule on PHFs. At the time, there was some confusion about the PHF protein constituents. Using well-characterized antibodies to various MAPs as well as PHF polyclonal antibodies, tau had recently been established as a major component of PHFs (see above and Mandelkow and Mandelkow 2011). However, monoclonal antibodies to neurofilament (NF) proteins also strongly immunolabeled PHF (Anderton et al. 1982), and these were widely applied to AD brain sections. This confusion about PHF components was clarified when Nobuyuki Nukina and co-workers found significant cross-reactivities of certain NF monoclonal antibodies with phosphorylated epitopes of tau (Nukina et al. 1987). In light of such observations, it became essential to show unambiguously that Ub was a component of PHF. In 1987, we finally obtained

definitive evidence using protein chemical techniques that Ub was integral to the PHF (Mori et al. 1987).

Ubiquitinated Tau in PHFs: K48-Linked Mono- and Polyubiquitin Modification

In the mid 1980s, the distinction between mono- and polyubiquitination was not yet clear, and the role of the multiubiquitin chain as a strong degradative signal was just emerging. A fraction of H2B was shown to be monoubiquitinated, and lymphocyte homing receptor was similarly found to be ubiquitinated, but the significance of monoubiquitination was entirely unclear. In our research on Ub in PHFs, we were unable to identify the Ub-conjugated protein by direct sequencing. It was most likely to be tau, but we had no definitive evidence. In the period 1989–1992, the Ihara laboratory was mostly involved in determining the phosphorylation sites on tau proteins in PHFs. We became aware that ion spray mass spectroscopy (MS) was an excellent method for determining phosphorylation sites on PHF-tau (Hasegawa et al. 1992). This provided an opportunity to reanalyze purified PHFs from AD cortex and identify the Ub-targeted protein.

We had previously raised antibodies to the carboxy-terminal region (Gly-76) of Ub, which was predicted to be conjugated with ϵ -amino groups of lysines of other proteins targeted for proteasomal degradation. However, despite repeated trials using these antibodies, we were unable to identify a particular HPLC peak containing the Ub carboxyl terminus. In our

hands, Ub reactivity was detected exclusively in the insoluble AD brain fractions displaying so-called “PHF smears” on immunoblots. However, Ub itself was not responsible for this smear pattern, because we also observed Ub-negative/tau-positive smears, in addition to Ub-positive/tau-positive smears (Morishima-Kawashima et al. 1993). The guanidine HCl-solubilized, Ub- and tau-reactive material purified from AD cortex by HPLC was subjected to peptide mapping using *Achromobacter lyticus* protease I (AP1), which is highly specific for Lys-X bonds. This yielded peaks corresponding to API-generated fragments of Ub (Fig. 2), in addition to carboxy-terminal fragments of tau, whereas virtually no fragments derived from the amino-terminal portion of tau were recovered. As expected, fragments U1–U7 (see Fig. 2) were found in our digests, but U8 (the carboxy-terminal portion of Ub) was undetectable at the expected position of the nonconjugated U8. Instead, we observed several unusual HPLC peaks that neither corresponded to U1–U8 nor occurred in the digests of Ub-negative/tau-positive smears. By protein sequencing and MS, we identified four Ub-conjugated sites on tau (Fig. 3) and further identified K48-linked Ub chains (Morishima-Kawashima et al. 1993). Crude estimates suggested that most of the products were derived from monoubiquitination and only a minority from polyubiquitination.

These Ub- and tau-reactive smears were recovered exclusively from the buffer-insoluble fraction of AD cortex, suggesting that ubiquitination might occur after tau deposits into

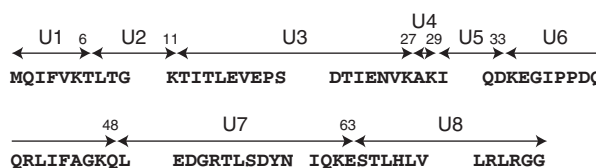


Figure 2. Schematic illustration of 76-residue ubiquitin. Ubiquitin has seven lysine residues and *Achromobacter lyticus* protease I (AP1) cleaves ubiquitin into eight fragments, U1–U8. G76 is conjugated with ϵ -amino groups of lysine in the target protein. K48-linked ubiquitin chain is a strong degradation signal. Besides this, K6-, 11-, 27-, 29-, and 63-linked multiubiquitin chains are found in the cell (Xu and Peng 2006). It is likely that each type of chain has a distinct role in the cellular metabolism.

Y. Ihara et al.

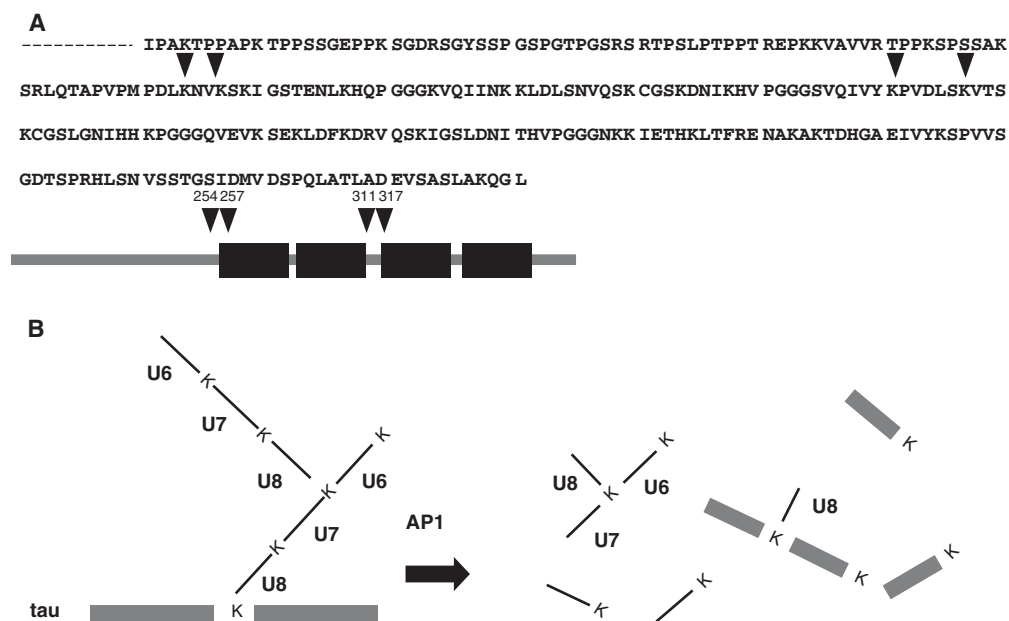


Figure 3. Ubiquitination sites on amino terminally processed tau. As shown in A, four ubiquitin conjugation sites were identified. These are located close to and in the microtubule-binding domain, K254, 257, 311, and 317 according to the numbering of the 441 amino acid human tau isoform. A size exclusion-purified paired helical filament PHF smear was further purified by reverse-phase HPLC, giving three broad (overlapped) peaks that existed only in smear fractions from AD brain. The last-eluting fraction contained a ubiquitin (Ub)-positive, tau-positive smear, which was subjected to AP1 digestion and reverse HPLC fractionation, followed by amino acid sequencing and mass spectrometric analysis. Several late-eluting peaks were found to contain branched fragments, mostly consisting of tau fragments and U8; a minority of U8 conjugated with Lys-48 of U6–U7, derived from the polyubiquitin chain (Morishima-Kawashima et al. 1993). Dysfunction of autophagic and endocytic pathways to lysosomes driven by relevant genes and other risk factors in Alzheimer disease (expressed on the *left* side of the diagram) causes or promotes pathophysiology critical to the development and progression of the disease (outlined on the *right* side of the diagram). See the text for further details.

aggregates. At that time, a simple model showed that cells conjugated Ub to abnormal proteins that were to be selectively degraded by the proteasome. If this was correct, minute amounts of abnormal PHF proteins tagged by Ub and targeted for the proteasome might be observed in the buffer-soluble fraction of cytoplasm, but we were unable to confirm this. However, recent work by Cripps et al. (2006) successfully used MC1 (a monoclonal specific for an abnormal PHF-like conformation of tau) to affinity purify soluble full-length tau from PHF-rich extracts of AD cortex and subject it to liquid chromatography–tandem MS (LC–MS/MS) analysis. The presence of K6, K11, and K48-linked polyubiquitinations—in

addition to monoubiquitination—was observed. Among these polyubiquitination sites, the K48-linked site was predominant. It is thus possible that a portion of MC1-reactive PHF-tau is in the soluble fraction and exists as free molecules in the cytoplasm, but this has not yet been confirmed.

Interestingly, immunocytochemical staining suggests that Ub exists in the mid-portion of neuropil threads (the largest source of PHF in the AD brain), whereas both ends of the threads are reactive for only tau, not Ub (Iwatsubo et al. 1992). This finding suggests that neuropil threads may extend at both ends: Tau may aggregate and deposit first, followed by its ubiquitination.

Ubiquitination of Other Neuropathological Inclusions

Following the discoveries about Ub and PHFs reviewed above, a variety of types of Ub–protein conjugates were detected in other neurodegenerative diseases. Although such findings were initially based solely on immunocytochemical evidence, we and others postulated that ubiquitination may be widely involved in the degradation of cytoplasmic proteins accumulating during aging and in a number of neurodegenerative diseases. Therefore, ubiquitinated inclusions may represent a failure of degradation by the Ub–proteasome system of neurons or glia. Early on, it was thought that Ub was associated with intermediate (10-nm) filaments in cells, because many inclusions seemed to consist of “altered” intermediate filaments (Lowe et al. 1988).

With the knowledge that DF2 recognizes Ub, we investigated whether DF2 stains other types of known intracellular inclusions. In the cortex, DF2 revealed fine dot-like stainings that had not been described before, and larger dot-like stainings were seen in the white matter, possibly derived from multivesicular and lysosome-related bodies. In addition, DF2 intensely labeled the classical granulovacuolar changes in the hippocampus in AD and other neurodegenerative disorders (Fig. 1, inset). We soon found that DF2 strongly stained cortical and brain stem Lewy bodies in brain sections from “diffuse Lewy body disease” (Kuzuhara et al. 1988), as originally described by Kenji Kosaka (1978), who proposed that some elderly subjects dying with dementia had many cortical Lewy bodies. In this dementia, Lewy bodies are abundant in cortical neurons, especially in the cingulate gyrus, in addition to their presence in the substantia nigra and locus ceruleus, their prototypical loci in Parkinson’s disease. The eosinophilic round bodies with clear margins in brainstem neurons and the somewhat indistinct, smaller round bodies in cortical neurons are sometimes difficult to recognize with conventional hematoxylin–eosin stains, but anti-Ub antibodies such as DF2 labeled them well. Furthermore, DF2 revealed a possible

Ubiquitin–Proteasome and Autophagy in AD

evolution of Lewy bodies: Diffuse cytoplasmic accumulation of Ub-positive material might be followed by its gradual coalescence into an amorphous perinuclear inclusion (Kuzuhara et al. 1988). DF2 and other Ub antibodies also labeled elongated neurites (now known as Lewy neurites) in and near the sites of Lewy bodies. Because Lewy bodies did not stain for tau, the target protein of ubiquitination had to be distinct. In 1997, α -synuclein was shown to be the principal component of Lewy bodies (Spillantini et al. 1997). The accumulated α -synuclein was then shown to be ubiquitinated (Hasegawa et al. 2002).

The DF2 immunoreactivity of Lewy bodies led us to search for similar DF2-positive inclusions, and we found that Lewy-like bodies in motor neurons in amyotrophic lateral sclerosis (ALS; Murayama et al. 1990a) and Pick bodies in Pick’s disease (Murayama et al. 1990b) were strongly reactive; the latter stained also for tau, whereas the former stained neither for tau nor α -synuclein. In this regard, the entity of frontotemporal lobar degeneration (FTLD) with Ub-positive/tau-negative inclusions was described later, and TDP43 was identified as the ubiquitinated protein in both this disorder and ALS (Neuman et al. 2006). It is noteworthy that the specific protein targets of ubiquitination that accumulate in insoluble deposits in distinct disorders have apparent major roles in the respective neurodegenerative processes: In AD, tau is ubiquitinated, in Parkinson’s disease and dementia with Lewy bodies, it is α -synuclein, and in ALS and FTLD-U, it is TDP-43.

Significance of Ubiquitinated Neurofibrillary Tangles

The discovery of tau ubiquitination in PHFs suggested that living (but presumably injured) neurons may mount an attempt to remove the abnormal inclusions (NFTs) by Ub tagging, although this apparently fails to efficiently remove the tangles. The UPS was believed to be involved principally in the turnover of short-lived proteins (cytosolic, nuclear, and endoplasmic reticulum [ER]), and especially

Y. Ihara et al.

tightly regulated transcription factors; for this process, the proteolytic system requires adenosine triphosphate (ATP). There was initially a view that long-lived proteins, including tau, may be degraded principally by lysosomes, the other major protein degradation machinery of the cell.

Ubiquitination occurs in three steps. The first step is activation of the carboxyl terminus of Ub by an E1 protein (i.e., a Ub-activating enzyme), which consumes ATP. Second, there is conjugation of the ATP-activated Ub to an E2 protein (i.e., a Ub-conjugating enzyme). Third, there is ligation of Ub with an ϵ -amino group of lysine in the target protein by an E3 ligase. E3 ligase binds both the target protein and the E2–Ub complex; thousands of substrate-specific E3s ensure selective protein tagging and degradation. An E4 enzyme catalyzes the polyubiquitination of the target substrate that is bound to the E2–E3 complexes. Proteins tagged with chains of four or more K48-linked multiubiquitins provide the strongest signal for degradation by the 26S proteasome, because a chain of at least four Ub moieties is required for substrate recognition by the 26S proteasome complex. Ubiquitinated target proteins bind

to the 19S cap (RP), which has a Ub binding site and ATPase activity, and this leads to cleavage of Ub moieties from the target by deubiquitinating enzymes, unfolds the polypeptides and sends them to the narrow channel of the 20S core particle. The narrow canal of 20S proteasome (~ 700 kDa) consists of heptameric stacks of four rings, with the inner two being composed of β subunits and the outer two of α subunits (Gallastegui and Groll 2010). Thus, the general structure of the 20S core particle is $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ (Fig. 4). These subunits have activities as trypsin-like, chymotrypsin-like, and peptidyl–glutaminyl hydrolyzing activities. The catalytic portions face the interior surface; during its passage, the target protein is processed to short peptides, mostly between six and 10 amino acids. Deubiquitinating activity attached to the 19S cap is essential for complete degradation; if this is blocked, degradation is inhibited, and ubiquitinated substrates accumulate in the cytoplasm.

The 26S proteasome is primarily a cytosolic enzyme, but it is also found in ER membranes and localized to nuclei. The former activity is known as ER-associated degradation. The latter (nuclear) activity is thought to be involved in

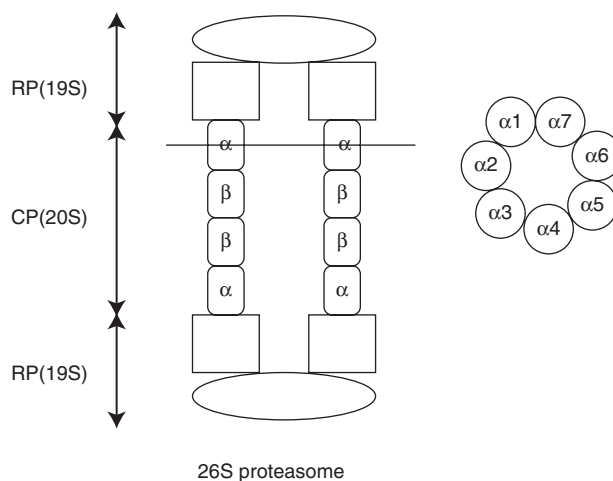


Figure 4. Schematic illustration of 26S proteasome. It consists of 19S regulatory particle (RP) and 20S core particle (CP). The narrow canal of CP consists of heptameric stacks of four rings, with the inner two being composed of β subunits and the outer two of α subunits. The image on the *right* shows a cross section at an indicated line (*left*). These subunits have trypsin-like, chymotrypsin-like, and peptidyl glutaminyl hydrolyzing activities. (Modified from Gallastegui and Groll 2010.)

the development of the ubiquitinated nuclear inclusions found in Huntington's disease and some types of spinocerebellar ataxia.

The question arises whether a fundamental impairment of proteasome function is involved in the formation of intracellular aggregates, in particular the NFT of AD. AD is the most common of numerous age-associated brain diseases, and the activity of brain proteasomes appears to decline with age (Keller et al. 2002). PHF have been associated with inhibition of the activity of the proteasome in a brain region-specific manner (Keller et al. 2000). That is, in areas where NFTs formed abundantly, including hippocampus and parahippocampal gyrus and superior and middle temporal gyri, proteasome activity (as assessed by chymotrypsin-like and postglutamyl peptidases) appeared to be most affected, whereas occipital gyri and cerebellum, which often have few or no NFTs, were least affected (Keller et al. 2000). Despite the decreased proteasomal enzymatic activity observed in AD brains, the levels of the α and β subunits were not decreased. This suggests that posttranslational modifications could cause the decreases in the activities of proteasome. Another study of AD brain tissues showed that hyperphosphorylated tau was bound to the proteasome, presumably to the 19S cap portion, and the more tau that was bound, the more that proteasomes appeared to be inhibited (Keck et al. 2003). In vitro experiments further showed that aggregated (recombinant) tau—but not nonaggregated (monomeric) tau—can inhibit the proteasome activity. The hyperphosphorylation of tau may not be related to suppression of proteasomes. Based on these various findings, it has been speculated that small aggregates of PHFs may bind to the cap portion of the 26S proteasome and inhibit its activity. This hypothesis is consistent with related observations made in other cell model systems (Bence et al. 2001). Taken together, the available evidence suggests that PHF-bearing neurons have defective activity of some of their proteasomes, which may contribute to the neurodegeneration. On the other hand, it seems unlikely that the decreasing activity of the proteasome leads to NFT formation. Aggregate formation itself may be a protective

Ubiquitin-Proteasome and Autophagy in AD

event in AD neurons and help extend their life spans, as compared with neurons not bearing NFTs (Gomez-Isla et al. 1997).

The currently prevailing view of the temporal involvement of Ub in PHF evolution is that the aggregation of hyperphosphorylated tau is followed by ubiquitination. This is consistent with previous immunocytochemical observations (Baner et al. 1991) and also with confocal microscopy reconstructions, suggesting that, in the neuropil threads of AD cortex, both growing ends of the threads contain full-length tau, whereas Ub is present at the mid portion, where the amino-terminal region of tau may have been processed (Iwatsubo et al. 1992). Similarly, in our biochemical studies, ubiquitinated tau was undetectable in the soluble fraction of cortex (but see Cripps et al. 2006), and only a portion of amino-terminally processed tau in the insoluble fraction was ubiquitinated (Morishima-Kawashima et al. 1993). The question remains why only a minority of the ubiquitin tagged to the processed tau in PHF is in the form of polyubiquitin chains, and what the significance is of the monoubiquitination that appears to represent the majority of ubiquitin found in PHFs (Morishima-Kawashima et al. 1993).

The Ubiquitin-Proteasome System in AD

Since ubiquitin immunoreactivity has been documented in a range of inclusion bodies, a role of the UPS in the pathogenesis of a number of neurodegenerative diseases, especially in AD, has been considered. Several molecules that are relevant to the UPS have been shown to be associated with AD. Let us first discuss UBB^{+1} , a mutant form of ubiquitin extending 19 amino acids at its carboxyl terminus which is generated owing to dinucleotide deletions of the ubiquitin mRNA through "molecular misreading" (van Leeuwen et al. 1998). UBB^{+1} protein accumulates in brains affected by AD and other diseases such as Pick's disease and Huntington's disease (Fischer et al. 2003). Because of the absence of the carboxy-terminal Gly-76, UBB^{+1} cannot ubiquitinate other proteins, but it is itself ubiquitinated efficiently. The

Y. Ihara et al.

resulting polyubiquitinated UBB⁺¹ cannot be degraded by proteasomes and impairs the UPS (Lam et al. 2000), which may induce neurotoxicity. Indeed, transgenic mice expressing UBB⁺¹ have an impaired UPS and show contextual memory deficits in both water maze and fear conditioning paradigms, without specific neuropathological findings (Fischer et al. 2009). Further, UBB⁺¹ can enhance the susceptibility of yeast to the toxicity of protein aggregates (Tank et al. 2009).

Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), which was originally identified as a deubiquitinating enzyme, has multiple functions, including as a ubiquitin ligase and a stabilizer of monoubiquitinated proteins (Setsuie and Wada 2007). Although UCH-L1 is genetically associated with Parkinson's disease (i.e., it is the PARK5 gene; Belin and Westerlund 2008), it has also been implicated in the pathogenesis of AD. A proteomic analysis has shown down-regulation and oxidative modification of UCH-L1 in the AD brain, and the levels of soluble UCH-L1 were inversely proportional to the number of NFTs (Choi et al. 2004). Moreover, administration of UCH-L1 can reverse the amyloid β -protein-induced synaptic dysfunction and memory loss in transgenic mice overexpressing APP and PS1 (Gong et al. 2006).

Next, ubiquilin-1 has been reported to be genetically linked to AD. Ubiquilin-1 interacts with both proteasomes and ubiquitinated proteins and regulates the proteasomal degradation of various proteins, including presenilin 1 (Haapasalo et al. 2010). An intronic polymorphism involving alternative splicing of exon 8 in the ubiquilin 1 gene (*UBQLN1*), which is genetically located near a well-established linkage peak for AD on chromosome 9q22, has been associated with increased risk for late-onset AD (Bertram et al. 2005). However, this finding has not been replicated by others (Smemo et al. 2006).

The earliest symptoms of AD are believed to be due to synaptic dysfunction, and in this context, numerous studies have established a significant role of the UPS in the regulation of synaptic plasticity. Although ubiquitin tagging was originally identified as a signal for protein

destruction by the proteasome system, ubiquitination is now known to be a posttranslational modification in which ubiquitins can serve as signals for protein localization and sorting in various physiological phenomena such as signal transduction, the cell cycle, transcription, DNA repair, and endocytosis. In particular, the critical role of the UPS in synaptic plasticity, learning and memory formation needs to be emphasized (Bingol and Sheng 2011). The role of the UPS in synaptic plasticity was first highlighted in *Aplysia*. During the induction of long-term facilitation in the snail, the regulatory subunit of cAMP-dependent protein kinase (PKA) is ubiquitinated and degraded by the proteasome, generating persistently activated PKA (Hegde et al. 1993). Activated PKA induces transcription of ApUCH (UCH-L1 in mammals), a deubiquitinating enzyme, which has been found to be critical for the induction of long-term facilitation (Hegde et al. 1997). It is now known that the UPS also regulates turnover of neurotransmitter receptors, protein kinases, synaptic proteins, transcription factors, and other molecules critical for proper synaptic plasticity, thus controlling synaptic strength and connections during brain development in mammals (Bingol and Sheng 2011). The UPS is also critically involved in learning and memory. Bilateral injection of lactacystin, a specific proteasome inhibitor, into the CA1 region of the rat hippocampus blocks long-term memory formation (Lopez-Salon et al. 2001). This and numerous related findings suggest that degradation of certain critical proteins by the UPS is required during long-term memory formation. One of these proteins is arc, a negative regulator of synaptic strength that promotes the internalization of AMPA receptors and is degraded via the E3 ligase, UBE3A (Greer et al. 2010).

Synaptic loss has long been documented in AD brain (Gonatas et al. 1967) and, as expected, is strongly correlated with the degree of cognitive impairment (Terry et al. 1991). In transgenic mouse models of AD, synaptic deficits have been detected prior to the formation of amyloid plaques (Hsia et al. 1999). Among the various molecular species of A β present in the brain, soluble oligomeric forms of A β are

arguably the most plausible candidates to impair synaptic function (reviewed in Walsh and Selkoe 2004). Soluble A β oligomers inhibit hippocampal long-term potentiation and alter memory and learning performance. They also facilitate long-term depression by, among other effects, disrupting synaptic glutamate uptake (Li et al. 2009). Recently, it has been shown that soluble A β oligomers isolated from AD cortex can induce tau hyperphosphorylation at AD-relevant epitopes and subsequent neurotic degeneration (Jin et al. 2011). Beyond an age-related reduction (Keller et al. 2002), proteasome activities decrease in AD in a brain region-specific manner, particularly in hippocampus, parahippocampal gyrus, superior and middle temporal gyri, and the inferior parietal lobule (Keller et al. 2000), areas that are especially critical for long-term memory formation. Moreover, soluble A β oligomers themselves can inhibit proteasomal activity (Tseng et al. 2008). Thus, it is possible that additive effects of A β oligomers and reduced proteasome activities in AD may accelerate synaptic dysfunction.

It is still unsettled how the degradation of tau is regulated under physiological conditions. It has been reported that tau is degraded by several major cellular degradation systems, including calpain, caspases, lysosomes, and proteasomes. As regards the proteasome, both the ATP-dependent 26S proteasome and the ATP-independent 20S proteasome have been reported to degrade normal, soluble tau (Cardozo et al. 2002; Zhang et al. 2005). However, administration of inhibitors of the proteasome has provided conflicting results: Treatment with lactacystin either did or did not suppress the degradation of tau (David et al. 2002; Brown et al. 2005a). On the other hand, it has been reported that the E3 ligase CHIP (carboxyl terminus of the Hsc70-interacting protein) binds to tau and is involved in the degradation of abnormal forms of tau, including insoluble tau and hyperphosphorylated tau, coordinately with Hsp70 (Petrucci et al. 2004; Dickey et al. 2006). As described above, it has also been found that the 20S proteasome interacts with tau aggregates (Keck et al. 2003). Collectively, such findings suggest that the UPS may be

Ubiquitin-Proteasome and Autophagy in AD

implicated more in the degradation of abnormal forms of tau than of normal, soluble tau.

As in the case of tau, an impairment of proteasome activity by protein aggregates is observed for other proteins, such as polyglutamine-expanded proteins, the prion protein, and A β (Bence et al. 2001). Notably, immunotherapy against A β in the 3xTg-AD mice, which reduces A β oligomers, reverses the proteasome deficits of these transgenic mice (Tseng et al. 2008). Thus, the accumulation of tau and of A β , forming the two major protein lesions of AD, impairs proteasome activity *in vivo*.

Despite the substantial indirect evidence just reviewed that associates UPS dysfunction with key features of AD, it remains unclear whether aberration in the UPS plays a causative or only a secondary role. Besides its key role in the UPS, ubiquitin is now known to function as a signal tag in the lysosome-autophagy system (see below). Indeed, many recent studies suggest the involvement of autophagy in the pathogenesis of AD. For example, immunocytochemistry showing the presence of K63-linked polyubiquitin in a fraction of the NFTs in AD cortex (Paine et al. 2009) suggests an active involvement of autophagy in the mechanism of AD.

PART II: THE AUTOPHAGY AND THE ENDOSOMAL-LYSOSOMAL SYSTEM IN AD

The Lysosomal Network

The endocytic and autophagic pathways share in common the role of delivering unneeded cellular materials to lysosomes for degradation and recycling to provide energy and new synthesis. Vesicular compartments in these pathways actively cross-talk and have common points of regulation (Simonsen et al. 2009; Settembre et al. 2011). Under pathological conditions, a deficit in one pathway often impedes functioning of the other. Therefore, it is reasonable to view these two pathways, which intersect at the lysosome, as comprising a lysosomal network (Fig. 5).

Although key to the survival of all cells, *endocytosis* supports unique neuronal functions, including aspects of synaptic transmission and

Y. Ihara et al.

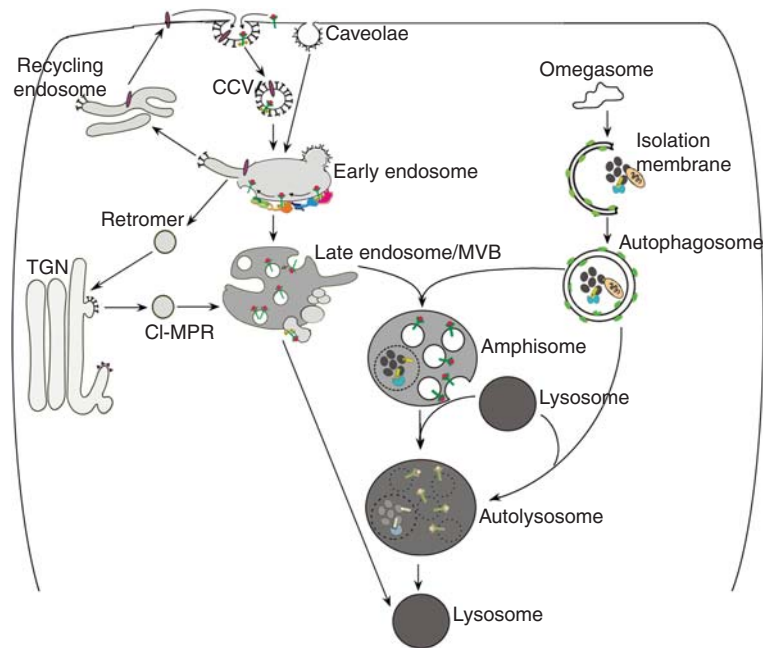


Figure 5. Schematic illustrating the endocytic and the autophagic pathways to the lysosome. See the text for details.

plasticity underlying memory and learning. Also, extracellular signals (e.g., growth factors) activate surface receptor complexes that, upon internalization, undergo retrograde axonal transport within “signaling endosomes” to promote neuronal survival and differentiation (Delcroix et al. 2003). That these vital processes are initiated in synaptic terminals at great distances from the perikaryon renders neurons particularly vulnerable to impairments of endocytic function and vesicular transport.

During the initiation of endocytosis, the invagination of plasma membranes into vesicles is mediated most often by the clathrin/adaptor protein complex, but also via caveolae or bulk macropinocytosis (Lim et al. 2011). Cargoes are delivered to early sorting endosomes through fusion mediated by the small GTPase Rab5 and additional Rab5 effector proteins (Fig. 5). Many cell surface proteins and lipids are returned to the plasma membrane via recycling endosomes, whereas other components are delivered to the Golgi by the retromer complex (Kelly et al. 2011). Still other cargoes reach

late endosomes when Rab7 and its effectors replace Rab5 and initiate further endosomal maturation (Poteryaev et al. 2010). An MVB is then created by inward budding of the surface membrane (Piper et al. 2001), thereby sorting cargoes into intraluminal vesicles. Ubiquitin, the crucial signal for efficient sorting of proteins into the MVB (Babst et al. 1997), initiates this process, which is mediated by a group of ESCRT complexes (endosomal sorting complex required for transport; Hurley 2010). Acid hydrolases, including cathepsins, are delivered from the trans-Golgi network (TGN) to MVBs/late endosomes by either of two mannose-6-phosphate receptors: cation-dependent 46 kDa MPR (CD-MPR) and cation-independent 215 kDa MPR (CI-MPR; Mullins et al. 2001). Proteolysis begins in late endosomes/MVBs but accelerates upon fusion with lysosomes, where cathepsins are activated by the highly acidic (pH 4.5–5.5) intraluminal environment. The acidic pH is achieved mainly through the ATP-dependent proton pump, vacuolar ATPase (Nishi et al. 2002), but is also modulated by a

group of chloride, sodium, calcium, and potassium transporters (Pillay et al. 2002).

Autophagy is the cell's principal degradative pathway for eliminating unwanted organelles and long-lived proteins and for clearing damaged, aggregated, or obsolete proteins (Wong et al. 2010). Autophagy refers to at least three processes by which intracellular constituents reach the lumen of MVBs or lysosomes for degradation: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy (Cuervo 2004; Levine et al. 2011). In CMA, cytosolic proteins containing a KFERQ motif (including proteins pathogenic in some neurodegenerative diseases) are selectively targeted by certain chaperones to the lysosomal lumen for degradation (Arias et al. 2011). Microautophagy involves the nonselective entry of small quantities of cytoplasm into lysosomes or late endosomes/MVBs when the limiting membranes of these compartments invaginate and pinch off small vesicles for digestion within the lumen (Sahu et al. 2011). Finally, macroautophagy mediates bulk or targeted degradation of cytoplasmic constituents. During macroautophagy, an elongated "isolation" membrane created from a preautophagosomal structure sequesters a region of cytoplasm to form a double-membrane-limited autophagosome (Fig. 5). Two ubiquitin-like protein conjugation pathways are known to coordinate this process (Ohsumi 2001). Sequestered material within autophagosomes is digested when lysosomes or late endosomes fuse with the outer membrane of the autophagosome (Gordon et al. 1988). Amphisomes formed by the fusion of autophagosomes with early endosomes or MVBs/late endosomes are especially important in neurons, where a considerable proportion of endocytosed cargo is directed to the autophagic pathway prior to being degraded by lysosomes (Larsen et al. 2002). This is especially true in axons (Lee et al. 2011). Induction of autophagy is generally controlled by the mTOR kinase (mammalian Target of Rapamycin), which is regulated by growth factors (especially insulin) and nutrient levels. Autophagy is constitutively active in neurons and is nonselective under nutrient deprivation conditions but may be

Ubiquitin-Proteasome and Autophagy in AD

selective when damage uncovers a molecular target on an organelle such as a mitochondrion (Youle et al. 2011), which initiates signaling and a ubiquitin-ligase-mediated chain of events that triggers selective sequestration of the organelle (Dikic et al. 2010; Weidberg et al. 2011).

Molecular Pathology of the Lysosomal Network

A continuum of pathological changes of the lysosomal network unfolds in neurons as Alzheimer disease progresses, including dysregulation of endocytosis, increased lysosome biogenesis and, later, progressive failure of lysosomal clearance mechanisms (Fig. 6; Nixon et al. 2006). These changes can be driven by known AD genetic and environmental risk factors, as discussed later. Endosome anomalies are the earliest specific pathology reported in AD brain tissue. Neuronal endosome enlargement, which is not characteristically observed in other major neurodegenerative diseases, develops in pyramidal neurons of the neocortex at a stage when plaques and tangles are restricted only to the hippocampus (Braak stage 2) and not in brains of similarly aged individuals free of AD-like hippocampal pathology (Cataldo et al. 1997, 2000). Similar endosomal anomalies develop gradually in Down syndrome brain, beginning decades before the appearance of classical AD pathology (Cataldo et al. 2000). Genes related to endocytosis, such as Rab5, Rab7, and Rab4, are among the earliest groups to show up-regulated transcription in AD (Ginsberg et al. 2010), and their corresponding proteins are abnormally recruited to endosomes, where they promote fusion and abnormal enlargement of early and late endosomes (Cataldo et al. 1997, 2008). This pattern is not seen in normal aging brain and is specific for AD among aging-related neurodegenerative diseases that have been studied (Cataldo et al. 2000).

As neuronal endosomes begin to enlarge, lysosomal biogenesis rises, as evidenced by lysosome proliferation and increased expression of acid hydrolases and other lysosome-related proteins (Nixon et al. 2006). Lysosome proliferation is followed by progressive enlargement

Y. Ihara et al.

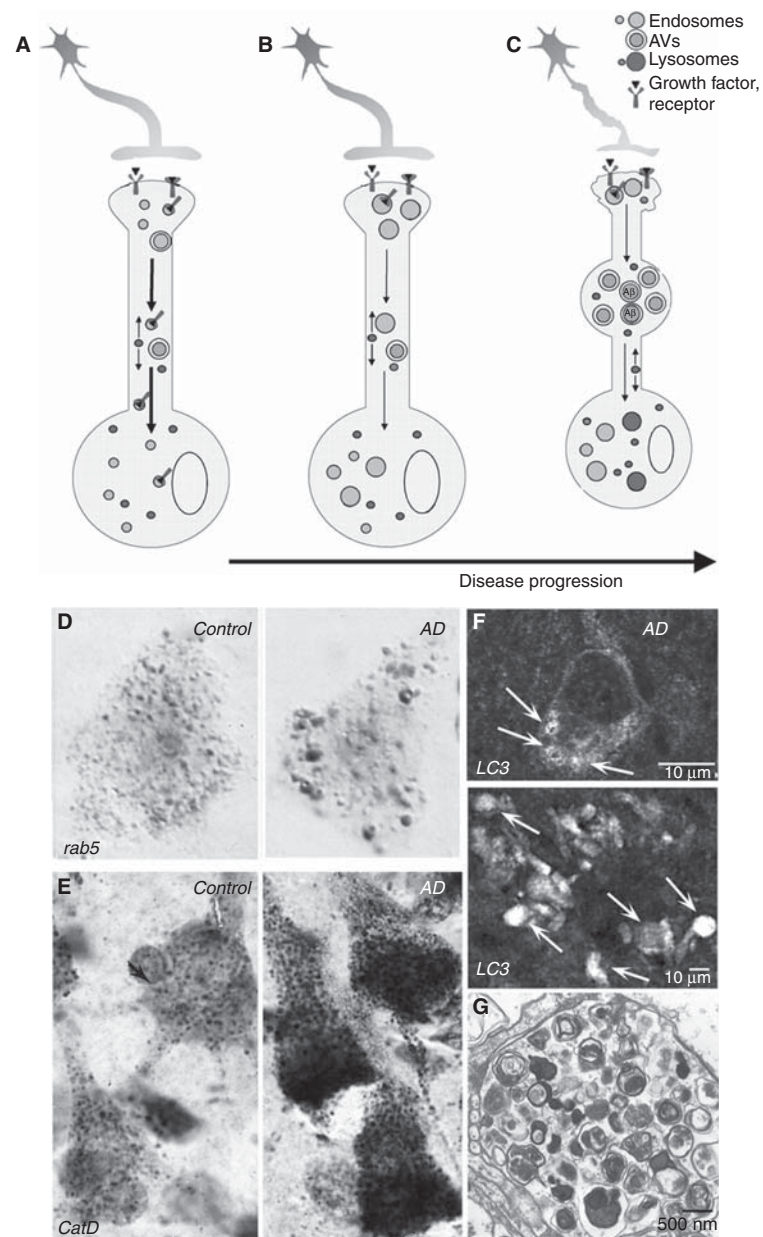


Figure 6. Progression of pathological changes in the lysosomal network in Alzheimer disease (AD). A normal neuron is depicted in *A*. At the earliest stages of AD (*B*), accelerated endocytosis, mediated in part by β CTF and/or by pathological activation of *rab5*, causes endosome enlargement (*D*; *rab5* immunocytochemistry), defective retrograde transport of endosomes and their neurotrophin cargoes, proapoptotic pathway activation, and cholinergic neurodegeneration. Initial up-regulation of lysosome biogenesis (*E*; cathepsin D immunocytochemistry) is followed by progressive failure of lysosomal proteolysis (*C*), which impedes autophagy and the axonal transport of late endosomes/MVBs and autophagy-related vesicular compartments. These compartments, containing incompletely digested protein substrates, selectively accumulate within neurons and especially dystrophic neurites (*C*), as depicted in *F* (LC3 immunocytochemistry) and *G* (ultrastructure of AVs in a dystrophic neurite). Failure of lysosomal proteolysis and autophagy in AD is caused by PS1 mutations in early-onset FAD and is also promoted by normal aging, oxidative stress, ApoE ϵ 4, intracellular A β , and other AD-related genetic and environmental risk factors.



and distortion of these compartments. Cathepsin-positive vacuoles massively accumulate within swellings of dystrophic neurites throughout the neuropil and in senile plaques as they develop (Cataldo et al. 1990, 1991). Ultrastructural investigations of AD brain biopsies have revealed that the most prevalent organelles by far within dystrophic swellings are autophagic vacuoles (AVs), representing all stages of the macroautophagic process (Nixon et al. 2005), including autophagosomes. This implies that the progression of autophagy is delayed or impaired, because AVs are relatively rare in normal brain. AV accumulations are not specific to the degenerative phenomena of AD; however, in AD brain, the extensive numbers of dystrophic neurites (Masliah et al. 1993; Schmidt et al. 1994), their characteristic marked distension, and the fact that they are predominantly filled with AVs distinguish the pattern and magnitude of this pathology from that of other aging-related neurodegenerative diseases (Benzing et al. 1993). In non-AD neurodegenerative disorders, many types of vesicles and cytoskeletal elements are relatively abundant, suggesting general axonal transport failure distinct from the selective transport deficits seen in AD (Lee et al. 2011a,b).

The profuse and selective accumulation of AVs in neurons in AD reflects a defect in the clearance of AVs by lysosomes rather than an abnormally elevated induction of autophagy. The accumulating AVs are mainly electron-dense autolysosomes, indicating that autophagosome-lysosome fusion is relatively competent. Similar autophagy pathology is observed when lysosomal proteolysis is inhibited (Ivy et al. 1984; Koike et al. 2005; Yang et al. 2008). That presenilin 1 mutations, which are a cause of early-onset familial AD, impede lysosome proteolysis and accelerate neuritic dystrophy also supports a primary role for failure of proteolytic clearance (Lee et al. 2010). Increased autophagy induction in AD could, however, compound the defect in AV clearance in AD. Transcription of many lysosomal genes and factors promoting autophagy is up-regulated, whereas negative regulators of autophagy are down-regulated (Lipinski et al. 2010), although

Ubiquitin-Proteasome and Autophagy in AD

not all studies support enhanced autophagy induction in AD (Pickford et al. 2008; Nixon et al. 2011).

AD Genetics Implicates the Lysosomal Network in Pathogenesis

Genetic factors that represent the strongest support for the amyloid/A β cascade hypothesis also act directly through A β -independent mechanisms to dysregulate the lysosomal network and contribute not only to A β accumulation but also to other critical aspects of AD pathogenesis (Fig. 7). Beyond its role as a component of γ -secretase, Presenilin 1 (PS1) is required for lysosome acidification, which is needed to activate cathepsins and other hydrolases that carry out digestion during autophagy (Lee et al. 2010). In the absence of PS1, the V0a1 subunit of v-ATPase is not N-glycosylated in the ER and is degraded before sufficient amounts can be delivered to autolysosomes/lysosomes to support lysosomal acidification. This function of PS1 is independent of the aspartyl protease activity of the molecule because proteolytically inactive PS1 mutated at one or both catalytic aspartates is able to restore normal lysosome acidification in PS1-deleted cells (Lee et al. 2010). Importantly, PS1 mutations also inhibit this process in fibroblasts from patients with familial AD (Lee et al. 2010), providing a basis for defective autophagy and the potentiation of autophagic-lysosomal and amyloid pathology and accelerated neuronal cell death observed in PS-FAD (Cataldo et al. 2004).

An additional copy of the *App* gene (genomic duplication) is sufficient to cause another form of early-onset FAD (Rovelet-Lecrux et al. 2006; Sleegers et al. 2006). *App* promoter polymorphisms that increase APP expression are also associated with early-onset AD (Athanasou et al. 2002). These findings support a longstanding hypothesis that the *App* gene on the trisomic copy of human chromosome 21 (HSA21) in Down syndrome (DS) is principally responsible for the invariant early development of AD in DS individuals (Margallo-Lana et al. 2004). In DS, the extra copy of *App* causes endocytic up-regulation and endosome pathology similar

Y. Ihara et al.

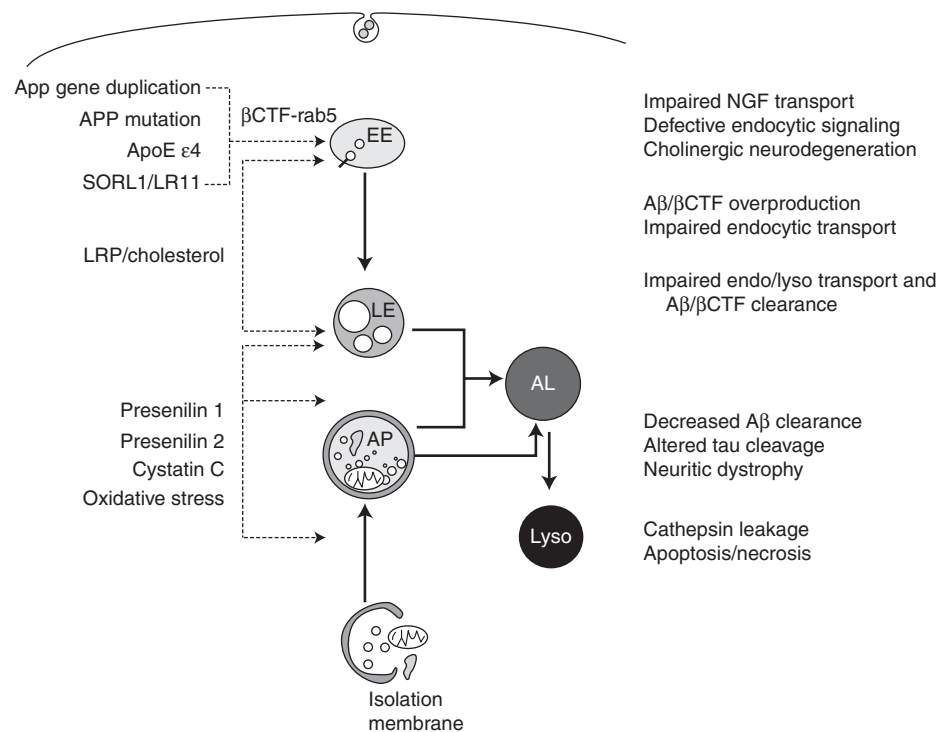


Figure 7. Dysfunction of autophagic and endocytic pathways to lysosomes driven by relevant genes and other risk factors in Alzheimer disease (*left side of the diagram*) causes or promotes pathophysiology critical to the development and progression of Alzheimer disease (*right side of the diagram*). See the text for further details.

to that seen at the earliest stages of sporadic AD, but beginning even earlier. Recently, these effects of increased *App* dosage were shown to be mediated specifically by the β -cleaved carboxy-terminal fragment of APP, β CTF (Jiang et al. 2010), which binds to a complex of signaling molecules on endosomes that pathologically activates rab5 (S Kim and RA Nixon, unpubl.). It is this abnormal rab5 activation that causes protein and lipid accumulation in endosomes, slowed lysosomal degradation of endocytic cargoes, endosome swelling (Cataldo et al. 2008), and disrupted retrograde transport of endosomes (S Kim and RA Nixon, unpubl.). Interactions between FAD-mutant forms of APP and APP binding protein (APP-BP1) on endosomes also initiate pathological rab5 activation, which was shown to promote a neuronal apoptosis cascade (Laifenfeld et al. 2007).

Acceleration of endosome pathology is also seen in individuals who inherit the $\epsilon 4$ allele of

APOE, a key mediator of neuronal cholesterol transport and the major genetic risk factor for late-onset AD (Cataldo et al. 2000). Moreover, among a very few additional pathological conditions associated with AD-like endosomal pathology are APP transgenic mice fed elevated dietary cholesterol and individuals with Niemann–Pick type C (NPC), a disorder of cholesterol homeostasis. High dietary LDL-cholesterol and overexpression of its receptor ApoE (particularly ApoE $\epsilon 4$) elevate β CTF levels (Ji et al. 2006; Cossec et al. 2010), and these levels are also elevated in NPC, DS, and AD, particularly in early-onset forms of AD caused by certain mutations of APP. The downstream consequences of β CTF-mediated rab5 activation and abnormal endosomal signaling for AD progression are many, as discussed later. Although less well characterized, a growing number of AD risk genes for late-onset AD identified through genome-wide screens and

association studies have direct links to endocytic regulation (Seshadri et al. 2010; Hollingworth et al. 2011; Hu et al. 2011).

The genetics of other neurological diseases further support a close connection between disruption of lysosomal network function and selective vulnerability to neurodegeneration (Nixon et al. 2008). Lysosomal storage disorders involving primary defects in lysosome function commonly exhibit prominent neurodegenerative phenotypes, including neuritic dystrophies closely resembling the ultrastructural morphologies of dystrophic neurites in AD and, in some of these disorders, neurofibrillary tangles as well as increased amyloidogenic processing of APP and diffuse β -amyloid deposits (Ryazantsev et al. 2007; Ohmi et al. 2009; Bellettato et al. 2010). Inherited defects in the endocytic pathway are also associated with neurodegenerative disorders at a high frequency (Nixon 2004; Nixon et al. 2008; Rusten et al. 2008; Zhang 2008).

Biology of Lysosomal Network Dysregulation in AD and Its Pathological Consequences

Neuritic Dystrophy

Endocytosis and autophagy are highly active at synaptic terminals and axons and generate large numbers of endosomes and autophagosomes, representing a considerable burden of retrograde organelle trafficking to the perikaryon (Overly et al. 1996). Transport and autophagy must, therefore, be especially efficient to prevent “traffic jams.” In healthy neurons, these mechanisms are, in fact, exceptionally efficient, as evidenced by a scarcity of autophagy-related intermediates, a high autophagic clearance capacity, and the rapidity with which axonal AVs accumulate when their clearance is impeded (Nixon et al. 2011), but they are also highly vulnerable to disruption (Boland et al. 2008; Cai et al. 2010; Lee et al. 2011a).

AVs and lysosomes constitute more than 95% of the organelles in dystrophic neuritic swellings in AD and AD mouse models, implying a cargo-specific defect in axonal transport, rather than a global one. Indeed, when lysosomal proteolysis is inhibited by blocking

Ubiquitin–Proteasome and Autophagy in AD

acidification or directly inhibiting cathepsins, axonal transport of autophagy-related compartments is selectively slowed and intermittently interrupted. These organelles then accumulate selectively within axon swellings that acquire additional AD-like features, including local cytoskeletal protein hyperphosphorylation and ubiquitin immunolabeling (Lee et al. 2011a). These observations suggest how PS1 mutations, which impede lysosomal acidification (Lee et al. 2010), may markedly accelerate and amplify neuritic dystrophy in AD.

Similar neuritic dystrophy eventually develops in all forms of AD and in mouse AD models where only FAD-causing mutant APP is overexpressed. In these cases, the mechanism is not yet clear. $A\beta$ administered directly into brains of wild-type rodents does not induce neuritic dystrophy, despite diffuse amyloid deposition (Frautschy et al. 1996, 1998), raising the possibility that other APP metabolites within neuronal compartments besides or in addition to $A\beta$ could be important to the development of neuritic dystrophy. In this regard, elevated β CTF levels induced by APP overexpression, elevated dietary cholesterol, or overexpression of its receptor ApoE (particularly ApoE ϵ 4) can up-regulate endocytosis and enlarge endosomes (Laifenfeld et al. 2007; Chen et al. 2010; Cossec et al. 2010), leading to impaired endosome retrograde transport (S Kim and RA Nixon, unpubl.). Accelerated endocytosis also increases protein and lipid accumulation in endosomes and slows lysosomal degradation of endocytic cargoes (Cataldo et al. 2008), leading to lysosomal instability and neurodegeneration, as discussed below.

Protein Clearance Failure and Amyloidogenesis

In APP transgenic mouse models of AD, undigested autophagy substrates including LC3-II, p62, and ubiquitinated proteins accumulate in neuronal AVs, establishing that autophagic protein turnover in lysosomes is impeded (Yang et al. 2011). This general failure to clear autophagy substrates affects clearance of various proteins relevant to AD pathogenesis, including

Y. Ihara et al.

A β , tau, and other factors, such as damaged mitochondria and activated caspases, that promote cell death (Yang et al. 2008).

Autophagy sequesters and digests unneeded or damaged organelles, some of which are APP-rich (Pickford et al. 2008). AVs are also enriched in APP substrates and secretases and, during autophagy, A β peptide is generated from APP (Yu et al. 2005), although it is subsequently degraded in lysosomes under normal circumstances (Heinrich et al. 1999; Bahr et al. 2002; Florez-McClure et al. 2007). Although less well studied as “A β degrading proteases” than the zinc metallopeptidase family (Guenette 2003; Eckman et al. 2005), cathepsins are considered an important route for A β /amyloid clearance (Mueller-Steiner et al. 2006; Nixon 2007; Butler et al. 2011) and human neurons may be particularly dependent on this mechanism (LeBlanc et al. 1999; reviewed in Saido and Leissring 2011). Chronic low-level stimulation of autophagy through peripheral administration of rapamycin or other agents (Tian et al. 2011), or enhancing lysosomal proteolysis selectively (Sun et al. 2008; Yang et al. 2011), can markedly diminish A β levels and amyloid load in APP transgenic mice, underscoring the importance of lysosomal clearance of A β .

Endocytic pathway up-regulation in AD stemming in part from pathological rab 5 activation generates higher levels of A β (Mathews et al. 2002; Grbovic et al. 2003) that must be cleared in part by lysosomes. Pathological rab5 activation, which in Down syndrome is dependent on β CTF generation (Jiang et al. 2010), can up-regulate endocytosis in a manner functionally equivalent to the elevated endocytosis associated with increased synaptic activity, which is considered a source of A β generation (Cirrito et al. 2008). An unknown amount of A β produced in endosomes reaches lysosomes directly or via production during autophagy (Nixon 2007), and accumulated AVs and MVBs appear to be the major intracellular reservoirs of A β immunoreactivity in AD brain (Takahashi et al. 2002; Yu et al. 2005). The acidic environment in lysosomes is particularly favorable for the initial stages of A β oligomerization (Peralvarez-Marín et al. 2008).

Tauopathy

Neurofibrillary tangles composed of tau proteins in a hyperphosphorylated state are rarely observed in abundance except in AD and a limited number of aging-related tauopathies. However, they are a prominent feature in at least two lysosomal disorders, NPC and mucopolysaccharidosis type IIB (MPS IIB; Ohmi et al. 2009), and in a mouse model of MPS IIB (Ryazantsev et al. 2007). In NPC, which arises from a defect in endosomal trafficking of cholesterol and the accumulation of unesterified cholesterol in late endosomes/lysosomes, additional features associated with AD are seen, including allele-selective influences of the APOE genotype on pathology and increased amyloidogenic processing of APP (Nixon 2004). The mechanisms explaining this connection are unclear, but tau has been found to be an autophagy substrate, at least when overexpressed (Berger et al. 2006). Incomplete chaperone-mediated autophagy of tau generates fragments that aggregate and are cleared by macroautophagy (Wang et al. 2009). Moreover, autophagy preferentially degrades a caspase-cleaved fragment of tau implicated in tau neurotoxicity (Dolan et al. 2010). Consistent with these findings, rapamycin induction of autophagy reduces tau pathology in the triple transgenic AD mouse model (Caccamo et al. 2010), whereas in other models, autophagic–lysosomal dysfunction amplifies tau pathology and tau neurotoxicity (Hamano et al. 2008; Khurana et al. 2010).

Synaptic Dysfunction

Pathological Rab5 activation driving endocytic dysfunction in AD may negatively impact long-term potentiation (LTP) and long-term depression (LTD) aspects of synaptic plasticity closely associated with learning and memory (Kessels et al. 2009). LTD induction requires surface removal of AMPA receptors driven by their Rab5-dependent internalization (Brown et al. 2005b). Conversely, deletion of the neuronal rab5 GEF, rin1, reduces rab5 activation, increases LTP induction in the amygdala, and enhances fear learning and memory, most likely by increasing surface levels of AMPA receptors

(Dhaka et al. 2003). Autophagy may also modulate synaptic plasticity, which involves structural remodeling of nerve terminals (Boland et al. 2006) and the trafficking and degradation of receptors and other synaptic proteins (Leil et al. 2004; Rowland et al. 2006).

Neurodegeneration

A close connection between lysosomal network dysfunction and mechanisms of neurodegeneration is well documented (McCray et al. 2008; Nixon et al. 2008; Bellettato et al. 2010; Cherra et al. 2010). Deprived of the cytoprotective functions of autophagy, neurons, which cannot dilute toxic protein buildup by cell division, are particularly vulnerable to potentially toxic mutant, oxidized and aggregated proteins and peptide fragments. An important possible outcome of the accumulation of the latter in lysosomes is increased membrane permeability and release of hydrolases into the cytoplasm, even from otherwise intact lysosomes (Kroemer et al. 2005). Cataclysmic disruption of lysosomal membranes releases hydrolases that act as both the trigger and executioner in rapid necrosis (Syntichaki et al. 2003; Kroemer et al. 2005), whereas slow release of cathepsins more likely operates through signaling pathways to trigger apoptosis (Kroemer et al. 2005).

By up-regulating endocytosis, high dietary LDL-cholesterol and overexpression of its receptor ApoE (particularly ApoE ϵ 4) elevate β CTF levels and increase delivery of A β 1–42 to lysosomes in cellular model systems (Ji et al. 2006; Cossec et al. 2010). Accumulation of A β 1–42, which is less efficiently degraded than A β 1–40 owing to its strong propensity to aggregate, causes leakage of lysosomal enzymes into the cytosol prior to other morphological signs of cellular toxicity in vitro (Yang et al. 1998; Glabe 2001). Consistent with these findings, strong overexpression of human A β 42, but not A β 40, in *Drosophila* neurons induces age-related accumulation of A β in autolysosomes and neurotoxicity (Ling et al. 2009). A β 42-induced neurotoxicity is further enhanced by autophagy activation and is partially rescued by autophagy inhibition. Expression of

Ubiquitin–Proteasome and Autophagy in AD

the ApoE ϵ 4 allele, but not ApoE ϵ 3, in mice administered a neprilysin inhibitor increases A β immunoreactivity in lysosomes and causes neurodegeneration of hippocampal CA1, entorhinal, and septal neurons (Belinson et al. 2008). ApoE ϵ 4 that trafficks to lysosomes more readily than ApoE ϵ 3, promotes leakage of acid hydrolases, and induces apoptosis in cultured neuronal cells by forming membrane-damaging intermediates in the low-pH environment (Ji et al. 2002).

Beyond influencing A β generation and toxicity, defective endosome functioning plays a crucial A β -independent role in the failure of retrograde NGF signaling that leads to basal forebrain cholinergic neuron degeneration in the Ts65Dn mouse model of DS (Cooper et al. 2001; Delcroix et al. 2004). Proteins known to play roles in both endocytosis regulation and cell survival decisions link the endosome dysregulation seen in AD to cell death cascade signaling (Vito et al. 1996, 1999; Missotten et al. 1999; Chen et al. 2000; Gout et al. 2000).

Remediating Lysosomal Network Dysfunction as AD Therapy

Recent evidence supports the value of targeting autophagy efficiency as a possible therapeutic strategy for AD. Peripheral administration of rapamycin to strongly stimulate autophagy substantially reduces amyloid deposition and tau pathology in both APP and triple transgenic mouse models of AD pathology (Caccamo et al. 2010; Spilman et al. 2010; Tian et al. 2011). Autophagy induction also has beneficial effects in transgenic models of several aging-related neurodegenerative diseases (Garcia-Arencibia et al. 2010). Stimulating lysosomal proteolytic efficiency in the TgCRND8 APP mouse model by deleting an endogenous inhibitor of lysosomal cysteine proteases (cystatin B) rescues lysosomal pathology, eliminates abnormal autolysosomal accumulation of autophagy substrates, including A β , decreases A β and amyloid deposition, and ameliorates learning and memory deficits (Yang et al. 2011). Similar therapeutic effects, including restoration of synaptic functions, are seen in APP mouse models after

Y. Ihara et al.

deleting cystatin C (Sun et al. 2008), by overexpressing cathepsin B (Mueller-Steiner et al. 2006), or by enhancing its activity (Butler et al. 2011). Collectively, these observations support the pathogenic significance of autophagic–lysosomal dysfunction in AD and specifically the importance of deficient lysosomal proteolysis.

CONCLUSION

As postmitotic cells, neurons are especially reliant on the lysosomal network to sort and clear normal and damaged proteins and support vital signaling functions. Not surprisingly, a defective endosomal–lysosomal pathway and certain autophagy-related genes are being linked with an unusually high frequency to neurodegenerative diseases, and the mechanistic relationship to neurodegeneration is becoming increasingly well established. Driven by the same genetic and environmental risk factors implicated in the A β /amyloid cascade hypothesis, a continuum of highly characteristic pathological changes in the lysosomal network evolves during AD, including dysregulation of endocytosis, increased lysosome biogenesis, and progressive failure of lysosomal proteolysis and autophagy. These deficits cripple neuronal functions critical for synaptic plasticity and neuron survival, lead to the hallmark neuritic dystrophy of AD, promote accumulation of toxic proteins—including A β , tau, activated caspases, and other cell death-promoting proteins—and initiate multiple neurodegenerative cascades. Remediation of lysosomal network dysfunction by several different approaches has been shown to have significant ameliorative effects on pathological and cognitive deficits in animal models of AD, underscoring the pathogenic significance of this dysfunction and the promise of therapeutic strategies targeting the lysosomal network in AD and other neurodegenerative disorders.

ACKNOWLEDGMENTS

The work of the Ihara laboratory on ubiquitin and PHF was carried out from 1985 to 1993, and Dr. Ihara thanks all laboratory members from

that period (Department of Clinical Physiology, Tokyo Metropolitan Institute of Gerontology; Department of Neuropathology, Faculty of Medicine, University of Tokyo). In particular, the preparation of the DF2 antibody and identification of ubiquitin in PHF was performed collaboratively with Dr Hiroshi Mori, Department of Neuroscience, Osaka City University Graduate School of Medicine. For the introduction and section on autophagy and the lysosomal system, the assistance of Nicole Piorowski and Corrinne Peterhoff in manuscript and figure preparation is gratefully acknowledged. Research from the Nixon laboratories is supported by the National Institute on Aging.

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Ubiquitin-Proteasome and Autophagy in AD



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Y. Ihara et al.



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Cold Spring Harb Perspect Med 2012; doi: 10.1101/cshperspect.a006361 originally published online April 4, 2012

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