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Reconsider Alzheimer's disease by the 'calpain–cathepsin hypothesis' —A perspective review

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ABSTRACT

Alzheimer's disease (AD) is characterized by slowly progressive neuronal death, but its molecular cascade remains elusive for over 100 years. Since accumulation of autophagic vacuoles (also called granulo-vacuolar degenerations) represents one of the pathologic hallmarks of degenerating neurons in AD, a causative connection between autophagy failure and neuronal death should be present. The aim of this perspective review is at considering such underlying mechanism of AD that age-dependent oxidative stresses may affect the autophagic-lysosomal system via carbonylation and cleavage of heat-shock protein 70.1 (Hsp70.1). AD brains exhibit gradual but continual ischemic insults that cause perturbed Ca^{2+} homeostasis, calpain activation, amyloid β deposition, and oxidative stresses. Membrane lipids such as linoleic and arachidonic acids are vulnerable to the cumulative oxidative stresses, generating a toxic peroxidation product 'hydroxynonenal' that can carbonylate Hsp70.1. Recent data advocate for dual roles of Hsp70.1 as a molecular chaperone for damaged proteins and a guardian of lysosomal integrity. Accordingly, impairments of lysosomal autophagy and stabilization may be driven by the calpain-mediated cleavage of carbonylated Hsp70.1, and this causes lysosomal permeabilization and/or rupture with the resultant release of the cell degradation enzyme, cathepsins (*calpain–cathepsin hypothesis*). Here, the author discusses three topics; (1) how age-related decrease in lysosomal and autophagic activities has a causal connection to programmed neuronal necrosis in sporadic AD, (2) how genetic factors such as apolipoprotein E and presenilin 1 can facilitate lysosomal destabilization in the sequential molecular events, and (3) whether a single cascade can simultaneously account for implications of all players previously reported. In conclusion, Alzheimer neuronal death conceivably occurs by the similar 'calpain-hydroxynonenal-Hsp70.1-cathepsin cascade' with ischemic neuronal death. Blockade of calpain and/or extra-lysosomal cathepsins as well as scavenging of hydroxynonenal would become effective AD therapeutic approaches.

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Abbreviations: Aβ, amyloid β; AD, Alzheimer's disease; ALLN, N-acetyl-Leu-Leu- Nle-CHO; apoE, apolipoprotein E; APP, amyloid precursor protein; APPLon, APP containing the London mutant γ-secretase site sequence; APPSwe, APP containing the Swedish mutant β-secretase site sequence; ASM, acid sphingomyelinase; BACE, β-Site APP cleaving enzyme; BMP, bis(monoacylglycero) phosphate; CA1, cornu *Ammonis* 1; CerS, ceramide synthase; CHIP, carboxyl terminus of the Hsc70-interacting protein; CNS, central nervous system; DHA, docosahexaenoic acid; ER, endoplasmic reticulum; ESCRT, endosomal sorting complexes required for transport; HNE, 4-hydroxy-2- nonenal (Hydroxynonenal); Hsc70, heat-shock constitutive protein 70; Hsp70.1, heat-shock protein 70.1; Lamp, lysosome-associated membrane protein; LMP, Lysosomal membrane permeabilization; MCI, mild cognitive impairment; MDA, malondialdehyde; MVBs, multivesicular bodies; NSAID, non-steroidal anti-inflammatory drugs; PI 3, phosphatidylinositol 3; rCBF, regional cerebral blood flow; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase dUTP and labeling; v-ATPase, v-type $[H^+]$-ATPase.

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1. Introduction

1.1. Drawback of the amyloid cascade hypothesis

More than one century ago, Dr. Alois Alzheimer (1907) described two important findings in the autopsy brain of a farmer's wife suffering from severe dementia; one is "*miliar foci which are caused by deposition of a peculiar substance in the cortex*" (now recognized as senile plaques), while another is "*very peculiar changes in the neurofibrils*" (now recognized as neurofibrillary tangles). Currently, Alzheimer's disease (AD) is the most common (~60%) form of dementia in the aged population. It is known to be a chronic neurodegenerative disorder with an insidious onset and progressive declines in cognition, memory, attention, and language. The risk of AD dramatically increases in individuals beyond the age of 70. AD begins with abnormal processing of amyloid precursor protein (APP), which leads to excess accumulation of amyloid β (Aβ) in the brain as senile plaques (Hardy and Selkoe, 2002). Such pathological process occurs gradually, and after a lag period presumably as long as two decades, neuronal dysfunction and degeneration become the dominant pathological processes (Jack et al., 2009). The majority of AD cases are non-genetic, sporadic forms, whereas merely less than 2.5% of all cases have a genetic origin. Compared with the healthy brain, the AD brain exhibits a marked shrinkage of the temporal and frontal lobes, and is histologically characterized by senile plaques and neuronal loss. Furthermore, especially cholinergic neurons are ultrastructurally characterized by neurodegeneration being associated with autophagic vacuoles (also called granulo-vacuolar degenerations), neurofibrillary tangles and synaptic loss. AD is arguably one of the most complex diseases of the aged human brain, but a cascade of molecular events leading to this disease remains grossly unknown for over a century.

The widely accepted risk factor for developing AD is Aβ deposition due to aging (Bi, 2010). More than 40 years ago, amyloid peptides were recognized by Glenner (1980a,b) as a major component of the amorphous plaque-like deposits in the damaged brains of AD patients. Since then, it became widely accepted that the amyloid cascade hypothesis best describes the pathogenic events causing Alzheimer neuronal death and leading ultimately to irreversible dementia. The amyloid cascade hypothesis (Glenner and Wong, 1984; Hardy and Selkoe, 2002; Citron, 2004; Herrup, 2010) claims that Aβ aggregates trigger a series of downstream events such as plaque deposition, tau hyperphosphorylation, inflammation, loss of synaptic structure and function, and death of susceptible neurons. This hypothesis postulates three key events that divert the brain into its pathophysiology. First, there has to be a precipitating injury such as vascular event, diabetes, head trauma, stress/life event, or risk factor genes. Second, a chronic inflammatory process is caused by elevated cytokines and chemokines that are produced by microglia, astrocytes, and endothelial cells. Third, a discrete change in the cell biological state of the brain occurs including widespread synaptic dysfunction, autophagy defects, selective neuronal loss, and attendant neurotransmitter deficits (Herrup, 2010).

In the human AD brain samples, specific variants of Aβ peptides, such as $A\beta_{1-42}$, N-terminally truncated $A\beta_{2/3-42}$, and pyroglutamate-modified $A\beta_{N3pE}$ peptides are prevalent. Especially, the most prominent N-terminal truncated Aβ peptide involved in human amyloid plaques is $A\beta_{N3pE}$ (Schieb et al., 2011). $A\beta_{N3pE}$ starts at residue 3Glu in the form of pyroglutamyl, and is more hydrophobic than other forms of Aβ, thus increasing the insolubility of Aβ (Saido et al., 1995; Kuo et al., 1997). N-terminally truncated Aβ species account for more than 60% of the Aβ peptides in early and later stages of AD amyloid pathology (Sergeant et al., 2003). In addition, $A\beta_{1-42}$ is also important in the pathogenesis of AD due to a higher propensity to aggregate (Jarrett et al., 1993; Jarrett and Lansbury, 1993). The amyloid cascade starting from depositions of these three peculiar Aβ is too complex web of interactions to view the whole diagram as sequential events, and this alone can hardly explain the pathogenesis of Alzheimer neuronal death. So far, Aβ deposition has been thought to be the primary event that triggers the pathological cascade in AD (Selkoe, 1998). However, the data in support of the amyloid cascade hypothesis have become questionable for the past decade (Robakis, 2010), because, (1) vast overproduction of Aβ peptides in the transgenic mice brain failed to cause severe neuronal loss and/or brain atrophy as seen in the human patients. Furthermore, (2) substantial amyloid deposits are sometimes (approximately 20–30%) detected in the brains of senior normal adults who lack any signs of clinical dementia. In addition, (3) the most serious drawback of the amyloid hypothesis is its inability to work therapeutically; i.e. removal of amyloid has contributed to the reversal of neither memory loss nor cognitive deficits. Nobody can explain how Aβ triggers the pathological cascade, how each initiating injury causes an inflammatory response, and how neuroinflammation alters brain cell physiology.

1.2. From apoptosis to necrosis

In the elderly, vascular risk factors such as arteriosclerosis, atrial fibrillation, coronary artery disease, hypertension, diabetes mellitus, smoking, insulin resistance, obesity or metabolic syndrome, are not only linked to brain aging and stroke but also significantly increase the risk of AD (Mattson, 2009; Stranahan and Mattson, 2012). Cerebrovascular disease is well-known to hasten the pathological processes leading to dementia. AD is three times likely to precipitate in the elderly after a stroke episode or a transient ischemic attack. Cerebral ischemia may promote Alzheimer type of changes in the aged brain, while 60–90% of AD cases exhibit variable cerebrovascular pathology (Kalaria, 2000). Pre-symptomatic vascular lesions such as cerebral amyloid angiopathy, microvascular degeneration, and periventricular white matter lesions are evident in almost all cases. Previous data suggest that almost 30% of AD patients bear evidence of cerebral infarction at autopsy (Premkumar et al., 1996; Kalaria, 2000). Although cerebral ischemia should have a causal connection to the development of Alzheimer neuronal death, there is no concensus about the role of ischemia in neurodegeneration. Both in vivo and in vitro experimental studies support the notion that

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ischemic injury can induce endogenous carboxyl terminal APP fragments containing the Aβ domain. Furthermore, the soluble Aβ peptides increase in brains of patients who succumb to cerebral ischemia (Kalaria, 2000).

In the AD brain, neuronal death occurs mainly in the hippocampus, entorhinal cortex, as well as frontal, parietal and temporal cortices. Because it occurs over a prolonged time of years, presumably a relatively small number of neurons are dying at any time point. Such a spatio-temporal pattern of neuronal death is considered characteristic of apoptosis and might contrast with necrosis (Mattson, 2004). Accumulating evidence from the molecular analyses strengthens involvement of apoptosis by activations of caspases, p53, Bax, Bad, etc. in the AD brains (Mattson, 2000; Eckert et al., 2003; McPhie et al., 2003). However, there are extremely very few, if no, morphologic evidence of apoptotic bodies in the degenerating neurons in the AD brain (Figs. 1 and 2). Instead, the degenerating neurons most frequently show a nucleus with vesicular and/or diffusely-condensed chromatin distribution (Fig. 1a). Apoptosis is one form of programmed cell death that involves changes in the cytoplasm, endoplasmic reticulum (ER), mitochondria and nucleus, whereas necrosis is another form of programmed cell death that involves changes especially in lysosomes (Yamashima, 2000). Importantly, the degenerating AD neurons are characterized by autophagic vacuoles (Fig. 1, 2c: yellow circles) and/or lysosomal lipidosis (multilamellar bodies as described later). Recently, it became clear that neuronal necrosis after ischemia/reperfusion also occurs by the lysosomal rupture in a very programmed manner (Yamashima et al., 1996, 1998, 2003; Syntichaki et al., 2002; Yamashima, 2000, 2004, 2012; Artal-Sanz et al., 2006; Yamashima and Oikawa, 2009; Zhu et al., 2012). Taken together, one can assume that apoptosis is not the only form of neuronal death in the AD brain, instead, necrosis might be implicated for its occurrence.

Compared to the age-matched control brains, the Alzheimer brain being analyzed by terminal deoxynucleotidyl transferase dUTP and labeling (TUNEL) method, showed a 3- to 6-fold increase of DNA fragmentation (Jellinger and Stadelmann, 2001). Obviously, such DNA fragmentation is too frequent to account for the continuous but gradual neuronal death in this slowly progressive disease. This is presumably because apoptosis is occurring mainly in the reactive microglia and oligodendroglia. Apoptotic neuronal death was extremely rare in the hippocampus; only one out of 2600–5650 hippocampal neurons displayed strong cytoplasmic labeling for activated caspase 3, and typical apoptotic morphology; i.e. reduction in cell size, chromatin condensation, and formation of apoptotic bodies (Jellinger and Stadelmann, 2001). Such extremely low incidence appears realistic in view of the protracted course of AD, compared to the very short duration (presumably less than one day) required for the completion of apoptosis. Intriguingly, the majority of TUNEL-positive neurons in the familiar AD bearing presenilin 1 mutation, showed ultrastructural features of not apoptosis but necrosis (Velez-Pardo et al., 2001). Neurons of the sporadic AD were characterized by endosomal/lysosomal abnormalities such as autophagosomes and/or autolysosomes containing multilamellar bodies (Nixon et al., 2005). Taken together, increased DNA fragmentation in the susceptible neurons does not necessarily

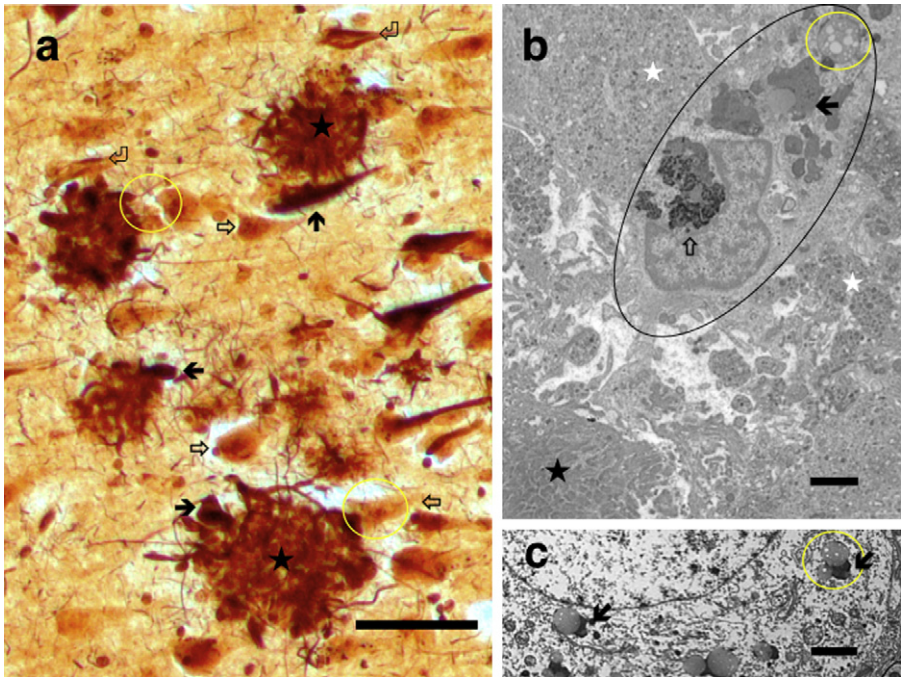

Fig. 1. Light and electron microscopic photographs of senile plaques and degenerating neurons in AD. (a) The senile plaques are indicated by the asterisks, while the neurofibrillary tangles are shown by curved white arrows. The senile plaques are fibrillar aggregates of Aβ being interspersed among clusters of dystrophic neurites, while the neurofibrillary tangles comprise of microtubule-associated protein tau that was abnormally phosphorylated and aggregated into paired helical filaments. Two types of the degenerating neurons, showing bright (white arrows) and dark (black arrows) neclei, are seen in the corona of the senile plaques (asterisks). The bright degenerating neurons are characterized by microvacuoles or granulo-vacuolar degenerations in the perikarya (yellow circles). Neuronal nuclei generally exhibit vesicular or condensed chromatin distribution without forming apoptotic bodies. The neuritic crown of senile plaques appears to incorporate these degenerating neurons and their neurites. One degenerating neuron in the corona of the senile plaque was observed by electron microscopy (b). Bar = 50 μm, Bielschowsky's silver stain. (b) Accumulation of autophagic dense vesicles (vacuoles) in the dystrophic neurites (white asterisks), are seen adjacent to an amyloid deposit (black asterisk). Since maturation (Fig. 7a) of autophagolysosomes and their retrograde transport (Fig. 5a) are impeded, a massive accumulation of these vesicles occur within dystrophic and degenerating neurites. Autophagic vacuoles and lysosomes constitute more than 95% of the organelles in the dystrophic neurites, implying a cargo-specific defect in the axonal transport (Fig. 5). The degenerating neuron (black circle) in the vicinity of the amyloid core contains abundant amyloid deposits (white arrow), lipofuscin granules (black arrow), and a multivesicular body (MVB; yellow circle). The latter is consistent with a granulo-vacuolar degeneration on light microscopy. Bar = 2 μm. (c) Ultrastructural appearance of MVBs (yellow circle) in the perikarya of the relatively healthy AD neuron. Please, note that small aggregates of amyloid (arrows) are usually adhering to the MVBs. Compare this with Figs. 2c and 6c. Bar = 2 μm (by the courtesy of Prof. Ralph A. Nixon, Nathan Kline Institute, New York).

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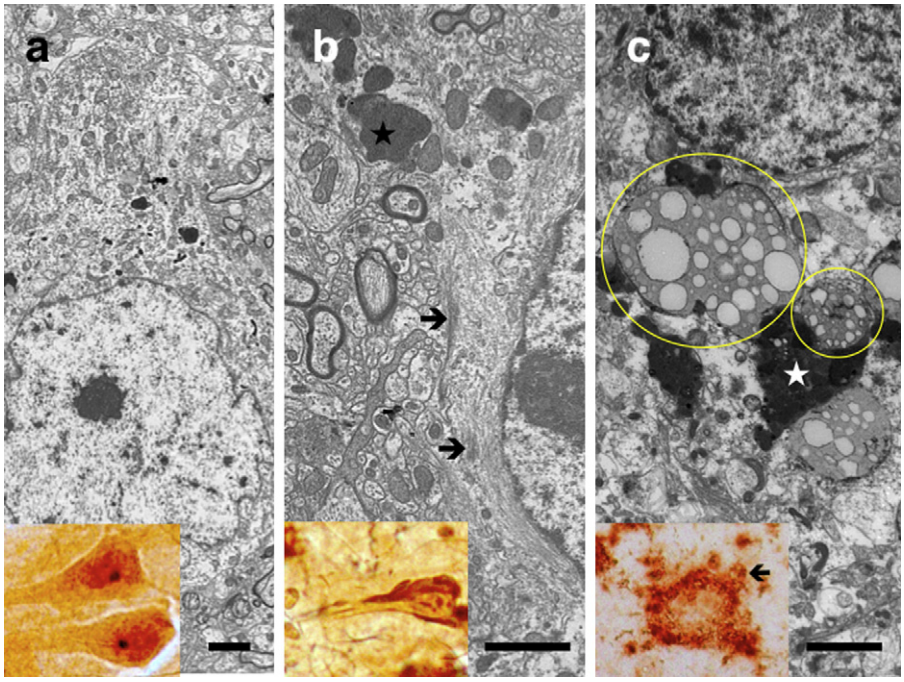

Fig. 2. Light and electron microscopic photographs of normal and AD neurons. (a) In the normal neuron of an age-matched control subject, the nucleus shows a vesicular chromatin condensation with a prominent nucleolus. The perikarya contains numerous organelles such as mitochondria, lysosomes and ER. EM: Bar = 2 μm, LM: Bielschowsky's silver staining. (b) The perikarya of the degenerating AD neuron contains neurofibrillary tangles (arrows) while the neurite is packed with numerous lipofuscin granules (black asterisk). EM: Bar = 2 μm, LM: Bielschowsky's silver staining. (c) The perikarya of the degenerating AD neuron shows 4G8-positive amyloid deposition and accumulation of autophagic vacuoles (also called granulo-vacuolar degenerations); both being adjacent to each other on light microscopy (insert; arrow). Here, the autophagic vacuoles are ultrastructurally revealed as MVBs (yellow circles). The irregular dense bodies (white asterisk) adjacent to the autophagic vacuoles can be identified as Aβ by comparing spatial relations on light and electron microscopy. Since MVBs cannot completely engulf Aβ peptide within their lumen, bundles of Aβ fibrils conceivably penetrate the vesicular membrane and transfer to the cytoplasm, as shown in Fig. 6c. Accordingly, the intracellular Aβ (white asterisk) is often localized in the close vicinity of MLBs. LM: Aβ immunostaining using 4G8 antibody (Covance, Princeton, New Jersey, USA), EM: Bar = 2 μm (by the courtesy of Prof. Ralph A. Nixon, Nathan Kline Institute, New York).

indicate implication of apoptosis, but reflects deficient DNA repair and accelerated DNA damage. Cells with these DNA disorders may show increased vulnerability toward hypoxia, oxidative stress, neurotoxins, etc. Accordingly, the intracellular cascade leading to neuronal death still awaits further elucidation in AD. Alternative mechanism of neuronal death should be considered so as to explain chronic and gradual progression of this disease.

1.3. Insights from ischemic to degenerative neuronal death

Axons, dendrites and spines are exposed to many different environmental conditions and signaling molecules. High content of APP, presenilins, and tau, as well as high metabolic and oxidative loads, may render synapses vulnerable. Synapses may be particularly susceptible to the adverse effects of aggregating forms of Aβ, because the latter may disturb neuronal functions by inducing oxidative stresses and disrupting cellular Ca^{2+} homeostasis. Oxidative stresses generate powerful oxidant, 4-hydroxy-2-nonenal (HNE; Fig. 4a) via peroxidation of membrane lipids such as linoleic and arachidonic acids. HNE is a strong electrophile and has the ability to readily adduct and damage proteins. Immunohistochemical analysis of the postmortem Alzheimer brains revealed increased protein modification by HNE (Sayre et al., 1997). When Aβ aggregation occurs close to the cell membrane, membrane-associated oxidative stress results in lipid peroxidation and the consequent generation of HNE may modify membrane transporters, receptors and ion channels (Mattson, 2009). Furthermore, oxidative modifications of tau by HNE can promote fibrillary aggregation and may thereby induce the formation of neurofibrillary tangles (Mattson, 2004). Inheritance of the apolipoprotein Eε4 allele (apoE4) is accepted to increase the risk for AD at an earlier age, because apoE4 lacking cysteine residues in its key sites cannot detoxify HNE (Pedersen et al., 2000; Tokuda et al., 2000). HNE is known to mediate toxic effects of Aβ to cultured hippocampal and cortical neurons. Identifying the mechanism of HNE-induced neuronal death pathways may be critical for understanding the pathogenesis of AD.

Recent research has underscored the roles of lysosomal pathways in AD, because deficiency or mutation in the presenilin 1 gene impairs maturation of the lysosomal proton pump (Lee et al., 2010). Neurons that degenerate in AD exhibit perturbed cellular Ca^{2+} homeostasis. As a mechanism of ischemic neuronal death, in 1998 Yamashima et al. formulated the '*calpain–cathepsin hypothesis*', according to which Ca^{2+}-activated cysteine protease 'μ-calpain' compromises integrity of lysosomal membranes and causes leakage of lysosomal acidic hydrolase 'cathepsins' into the cytoplasm (Yamashima et al., 1998). Calpain activation and/or HNE-induced carbonylation of heat-shock protein 70.1 (Hsp70.1; simply Hsp70, also called Hsp72 or HSPA1 indicating a major protein of the human Hsp70 family) have been confirmed not only in the ischemic monkey brain (Sahara and Yamashima, 2010) but also in the Alzheimer brains (Saito et al., 1993; Taniguchi et al., 2001; Sultana et al., 2010). Recent data advocate for a definite role for Hsp70 not only as a chaperone protein for damaged proteins but also as a guardian of lysosomal integrity (Kirkegaard et al., 2010). Accordingly, failure of lysosomal autophagy and proteolysis is driven by calpain-mediated cleavage of carbonylated Hsp70.1, that is promoted by chronic mild hypoxia and Aβ deposition due to aging, environmental risk factors and resultant oxidative stresses, and apoE4's incapability of detoxifying HNE. Deficiency of presenilin 1 disturbs acidification necessary for the recycling of proteins and lipids which may cause an enormous storage of undigested materials within autophagic vaculoes.

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Although the hallmark pathologic feature such as senile plaque deposition (Fig. 1a) has been considered mediators of neurotoxicity in AD, recent findings have raised doubts about the amyloid cascade hypothesis (Bi, 2010). Aβ deposition, oxidative stresses, inflammatory reactions, and AD-related gene mutations are accepted to be possible causative factors. However, no single cascade reported previously can simultaneously account for implications of all of these. This review proposes such a concept that long-standing, mild cerebral ischemia initiates 'calpain-mediated cleavage of carbonylated Hsp70.1', and the resultant lipidosis, membrane destabilization, and/or rupture of lysosomes are the main causes of programmed neuronal necrosis in AD. Because most cases of AD are sporadic and late-onset, the most important risk factor should be age-related lysosomal destabilizations. Primary lysosomal dysfunction in congenital neurodegenerative diseases is well known, however, the possibility was only recently recognized that neurodegeneration secondary to age-related dysfunctions in lysosomal resident and/or interacting factors may also lead to acquired neurodegenerative diseases (Yamashima, 2012). Here, the author discusses how each of the common Alzheimer's susceptibility genes and other well-known risk factors promoting AD contribute to lysosomal dysfunction, destabilization, and rupture.

2. Possibility of Hsp70.1 cleavage in Alzheimer's disease

2.1. Calpain activation in the Alzheimer brain

Intriguingly, Dr. Alois Alzheimer (1907) described that "*besides storage of peculiar material in the cortex, one sees endothelial proliferation and also occasionally neovascularization*". Here, we should note that ischemic pathology was already evident in his first AD case. Alzheimer described microvascular changes, in terms of "*endothelial proliferation*" and "*neovascularization*". Many years later, Fischer et al. (1990) also confirmed a striking and statistically significant reduction in the vascular net density specifically in the basal forebrain region and the hippocampus of AD brains. In the last two decades, the contribution of vascular factors to the etiology of AD has become a rapidly extending research field. Both Aβ- and oxidative stress-induced inflammatory damages to small blood vessels and the resultant reduction of the regional cerebral blood flow (rCBF), were thought to be critical for the occurrence of Alzheimer neuronal death (Fig. 3a–c) (Marchesi, 2011).

The majority (70–90%) of AD patients show cerebral amyloid angiopathy which narrows the vessels and causes cerebral ischemia (Farkas and Luiten, 2001; Cullen et al., 2006; Hardy and Cullen, 2006; Smith and Greenberg, 2009). There is a general

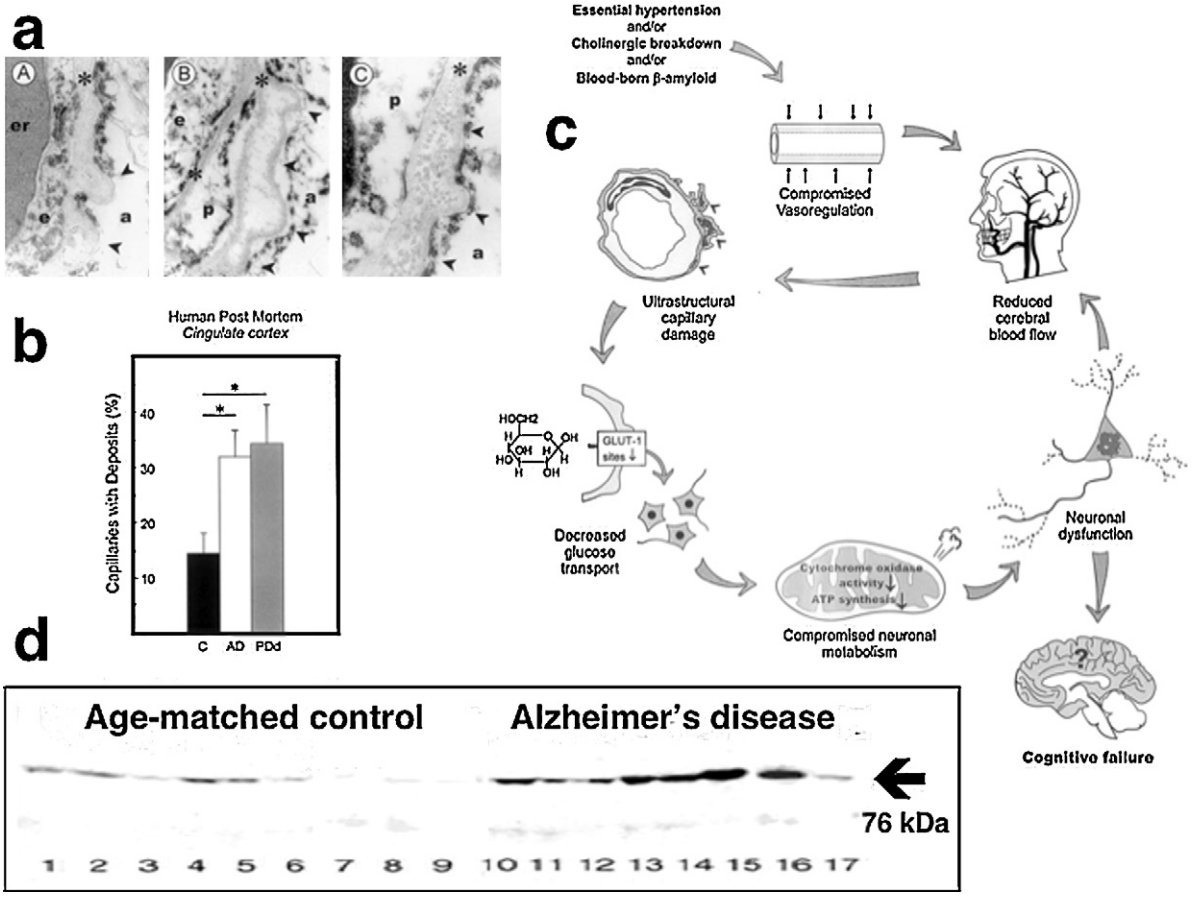

Fig. 3. The role of age-dependent capillary changes in AD neurodegeneration. AD patients often show amyloid pathology in their small vessels, which impairs the blood-brain barrier. Inflammatory reactions, ROS, resultant Aβ depositions, and apoE4 inappropriately collaborate to damage capillaries as the first step of cerebral ischemia. (a) In the patients with AD and Parkinson's disease with dementia as well, age-related, degenerative capillary changes occur at the basement membrane. Aβ may accumulate either in a non-fibrillary form or delicate fibrils in the capillary basement membrane (cited from Prog Neurobiol 64, 575–611, 2001). (b) The percentage of cerebral capillaries with basement membrane deposits and/or fibrosis in three different conditions. C, non-demented control; AD, Alzheimer's disease; PDd, Parkinson's disease with dementia. $^{*}P < 0.05$ (cited from Prog Neurobiol 64, 575–611, 2001). (c) Basement membrane pathology (Fig. 3a) will physically hinder passive transport of glucose, electrolytes and nutrients from blood into the brain parenchyma. Reduction of the cerebral microcirculation may enhance synaptic loss and neurodegeneration especially of cholinergic neurons, that lead to cognitive deficits. Then, chronic hypertension may accomplish its detrimental effects on cerebral vessels via inducing cerebral hypoperfusion (cited from Prog Neurobiol 64, 575–611, 2001). (d) The calpain activation in the AD brains occurs approximately seven-fold more intense, compared to the age-matched, non-demented controls. Arrow indicates 76 kDa bands indicating activated μ-calpain (cited from FEBS Lett 489, 46–50, 2001).

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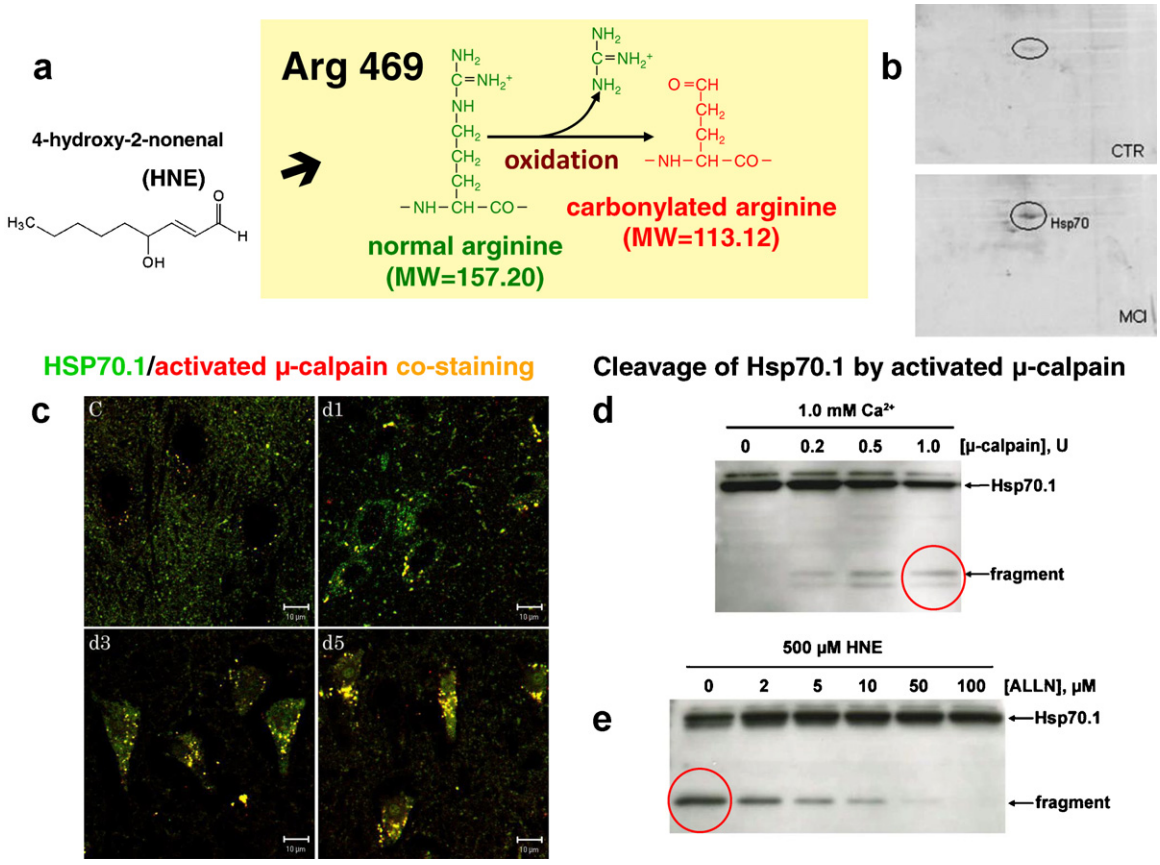

Fig. 4. 4-hydroxy-2-nonenal (HNE)-mediated carbonylation and calpain-mediated cleavage of Hsp70.1 in the monkey hippocampus. (a) Chemical structure of HNE being generated from $\omega = 6$ polyunsaturated fatty acids such as linoleic and arachidonic acids. In response to HNE being generated by the oxidative stress during the reperfusion, carbonylation occurs at the key site, Arg469 of Hsp70.1 in the CA1 tissue. A decrease of the molecular weight from 157.20 to 113.12 indicates an insult by the oxidative stress (cited from Free Radic Biol Med 46, 1472–1477, 2009). (b) Two-dimensional carbonyl immunoblots of the human brain samples after immunoprecipitation with anti-Hsp70 antibody, shows upregulation of carbonylated Hsp70 in the patient with mild cognitive impairment (MCI), compared to the control subject (CTR) (cited from Antioxid Redox Signal 12, 327–336, 2010). (c) Hsp70.1 (green) and activated μ-calpain (red) were shown to be colocalized by immunohistochemistry in the monkey CA1 neurons, especially on days 3 (d3) and 5 (d5) after transient ischemia. Scale bar = 10 μm. (d) In vitro cleavage of Hsp70.1 being involved in the normal CA1 tissue after incubation with 0.2, 0.5, and 1.0 U of activated μ-calpain. (e) Dose-dependent blockade upon calpain-mediated cleavage of carbonylated-Hsp70.1 by HNE, using a specific calpain inhibitor N-acetyl-Leu-Leu-Nle-CHO (ALLN). Please, note that Hsp70.1 cleavage by calpain occurs more drastically following HNE-mediated carbonylation (Fig. 5d and e: red circles).

consensus on a lower (approximately between 80 and 90% compared to the age-matched controls) cerebral perfusion in AD patients, and reduction of the rCBF was proportional to the degree of cognitive decline (Farkas and Luiten, 2001). Nerve terminals from the cholinergic neurons of the basal nucleus of Meynert interact with astrocytic end feet of the blood-brain barrier via muscarinic acetylcholine receptors (Vaucher and Hamel, 1995; Farkas and Luiten, 2001). Accordingly, a marked reduction predominantly of cholinergic neurons occurs in the basal forebrain (septum and nucleus basalis of Meynert) in advanced stages of AD that was associated with long-standing cerebral hypoperfusion (Wilcock et al., 1982; Whitehouse et al., 1983). Whereas the substantial contribution of the cholinergic pathways to cognitive functions is widely accepted, recently the striking coincidence of cholinergic neurodegeneration in the nucleus basalis and the reduced rCBF has also gained more attention in AD patients (Fig. 3c) (Farkas and Luiten, 2001).

The Alzheimer brains bear vascular pathology being capable of inducing cerebral infarcts, microinfarction, white matter changes related to small vessel disease, and even hemorrhages (Kalaria, 1996, 2000; Kalaria and Ballard, 1999). Since patients with cerebrovascular disease or vascular dementia often bear AD pathology at autopsy even though absence of pre-existing AD symptoms, there should be a causal connection between cerebral ischemia and AD (Kalaria, 2000). It is important to point out that in the early stage of AD, damaged vessels are at the capillary, arteriole, and venule level, not at the large muscular artery level (Fig. 3a and b). Damaged microvasculature creates ischemic conditions that might be initially confined to small areas of brain tissue, possibly affecting only a small number of neurons (Marchesi, 2011). Although stroke and severe cardiovascular diseases had been normally considered exclusion criteria for the clinical diagnosis of AD, the contribution of cerebral ischemia to AD neurodegeneration is nowadays apparent (Fig. 3c).

Calpain (EC 3.4.22.17) is an intracellular, non-lysosomal, Ca^{2+}-dependent, papain-like protease. For the half-maximal activity, μ-calpain requires micromolar (3–50 μM) levels of Ca^{2+}, whereas m-calpain requires nearly millimolar (400–800 μM) levels of Ca^{2+}. In the hippocampus, μ-calpain is localized at the pyramidal and granular neurons, whereas m-calpain is localized at the interneurons (Rami, 2003). Calpain participates in various Ca^{2+}-mediated signaling pathways by modulating activities and/or functions of other proteins. It is also responsible for the turnover of substrates being related to the cell mobility, and cell cycle progression (Suzuki et al., 2004). Physiologically, calpain is present in the cytosol as inactive precursors, but extreme conditions often result in overactivation of calpain, and activated calpain transfers to the membrane fractions. Not only cerebral ischemia (Yamashima et al., 1996, 1998, 2003; Yamashima, 2000, 2004) but also wild-type APP (Kuwako et al., 2002) and Aβ (Reifert et al., 2011)

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are known to induce a significant activation of calpain. One should note that the AD brains show as high as 7-fold μ-calpain activation, compared to the age-matched control brains (Fig. 3d) (Taniguchi et al., 2001).

In AD, μ-calpain was suggested to be involved in the processing of not only APP (Siman et al., 1990) but also tau proteins (Mercken et al., 1995; Yang and Ksiezak-Reding, 1995). Activated calpain was found to co-localize with neurofibrillary tangles, senile plaques, and dystrophic neurites (Saito et al., 1993). An inflammatory response accompanies tissue damage in many brain diseases, and AD is unique in the intimate association found between chronic inflammation and disease (Herrup, 2010). Epidemiological evidence shows that long-term use of high doses of non-steroidal anti-inflammatory drugs (NSAID) lowers the lifetime risk of AD by 30–60% (McGeer et al., 1996; Stewart et al., 1997; Vlad et al., 2008; Herrup, 2010). This is presumably because NSAID such as indomethacin and NS-398 [N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide]) can inhibit not only cyclooxegenase enzymes but also calpain activity (Raveendran et al., 2008; Silver et al., 2010). Although calpain has been suggested to be one of the principal effectors of the pathogenesis of AD (Nixon et al., 1994), its exact role in the Alzheimer neurodegeneration still remains unclarified.

2.2. Increment of a key substance, 'hydroxynonenal'

The brain is particularly susceptible to oxidative stresses because of its high content of peroxidizable polyunsaturated fatty acids, high oxygen consumption, high levels of free radical-inducing iron/ascorbate, and relatively low levels of anti-oxidant defense systems (Floyd, 1999; Poon et al., 2004a,b; Halliwell, 2006). Reactive oxygen species (ROS)-mediated oxidative stresses play a crucial role in the age-related decline of brain function as a result of the oxidation of nucleic acids, lipids, and proteins. Actually, proteins have many amino acid residues that are more susceptible to the oxidative stresses than deoxyguanosine in DNA. The pathophysiological meaning of variously oxidized protein molecules has been discussed not only in AD but also in aging, atherosclerosis, cataracts, glaucoma, cerebral ischemia, etc.

Furthermore, ROS have the potential to chemically modify membrane lipids, as shown by elevation of lipid oxidation products in the AD brains (Singh et al., 2010). Lipid peroxidation leads to the formation of a number of aldehyde by-products, including HNE, malondialdehyde (MDA), and acrolein (Esterbauer et al., 1991; Schneider et al., 2001). The most abundant aldehydes are HNE and MDA, while acrolein is the most reactive. Marked increase of HNE was reported in the hippocampus as well as in the superior and middle temporal gyri of patients with mild cognitive impairment (MCI) or early AD, compared to age-matched healthy individuals (Williams et al., 2006). Elevations in the free HNE concentration have been detected in the cerebrospinal fluid of AD patients (Lovell et al., 1997). This suggests that HNE is diffusible in the brain and can therefore modify proteins other than those located at the site where HNE was generated (Pedersen et al., 2000). HNE application can in vitro mimic AD pathology, including modification of tau (Takeda et al., 2000; Liu et al., 2005) and Aβ (Murray et al., 2007), inhibition of the glucose transporter (Reagan et al., 2000), and disruption of microtubules (Gadoni et al., 1993).

HNE is a major polar lipophilic secondary peroxidation product which can be generated from ω-6 polyunsaturated fatty acids such as linoleic and arachidonic acids when cooked at frying temperature (Esterbauer et al., 1991; Esterbauer, 1993). Abundant linoleic acid is involved in vegetable oils; for example, about 60% in corn oils and about 54% in soybean oils (Sonntag, 1979). Vegetable oils are worldwide used for frying, because of the convenience and low cost, however, these oils are especially susceptible to oxidation at high temperature (Kilgore, 1969; Kilgore and Bailey, 1970; Kilgore and Windham, 1973; Chang et al., 1978; Gere, 1982). Continuous or intermittent exposure of the vegetable oils to the frying temperature (185–220 °C) steadily accerates the formation of HNE (Seppanen and Csallany, 2006), because its formation is highly affected by the concentration of linoleic acid involved in the oils (Han and Csallany, 2008). It is widely accepted that HNE contributes to the development of neurodegeneration (Mattson, 2009), but the underlying mechanism has been unknown until recently. In studies on the role of lipid peroxidation in the pathogenesis of ischemic neuronal death, it was shown that Hsp70.1 is carbonylated by HNE (Oikawa et al., 2009; Yamashima and Oikawa, 2009; Sahara and Yamashima, 2010). This finding is of particular interest in relation to Alzheimer neuronal death as apoE genotype modifies its risk by detoxifying HNE (Pederson et al., 2000). It is well known that non-demented individuals carrying an apoE4 allele, a common Alzheimer's susceptibility gene (Corder et al., 1993; Strittmatter et al., 1993a,b), have cerebral glucose hypometabolism as compared to apoE4 non-carriers, and show progressive cognitive deterioration, correlating with the reduction of glucose metabolism (Small et al., 1995; Reiman et al., 1996; Small et al., 2000; Reiman et al., 2001, 2004).

Fluorodeoxyglucose-positron emission tomography studies have shown that AD patients have abnormally low cerebral metabolic rates for glucose in the posterior cingulate, parietal, temporal, and prefrontal cortices. Furthermore, carriers of the apoE4 allele have similar brain abnormalities already in young adulthood, several decades before the possible onset of dementia (Reiman et al., 2004). Furthermore, carriers of the apoE4 allele are associated with up to half cases of late-onset AD (Corder et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993a,b). The E4 isoform of apoE is known to be associated with increased Aβ aggregation, less protection against Aβ-induced oxidative neurotoxicity, less efficient repair of neurons and synapses, less protection against the hyperphosphorylation of the microtubule-associated protein tau, and the formation of neurofibrillary tangles (Mahley, 1988; Strittmatter et al., 1993a,b, 1994a,b; Wisniewski et al., 1994; Miyata and Smith, 1996).

The highest expression of apoE is in the liver, followed by the brain; neurons and astrocytes are the major cell types that express apoE in the brain (Pitas et al., 1987; Xu et al., 1999; Grehan et al., 2001; Xu et al., 2006). ApoE plays a key role in lipid transport throughout the body, and in the brain it is involved in the maintenance and repair of neurons (Mahley, 1988; Weisgraber and Mahley, 1996). Cholesterol being released from apoE-containing lipoprotein particles, is used to support synaptogenesis and synaptic connections (Pfrieger, 2003). While many putative susceptibility genes for AD have been reported to date, polymorphism in the apoE gene is the only confirmed genetic risk factor for sporadic AD; with the ε4 allele being an AD risk factor and the ε2 allele being protective (Corder et al., 1993; Strittmatter et al., 1993a,b). However, the mechanism by which apoE polymorphism influences occurrence and progression of late-onset AD has yet to be proven. The three common isoforms of apoE comprise of apoE2 (cys112, cys158), apoE3 (cys112, arg158), and apoE4 (arg112, arg158). The differences by only one or two amino acids at residue 112 or 158 greatly alter apoE structure and function (Mahley et al., 2006).

The risk for AD increases 2∼3-fold in individuals with one ε4 allele while about 12-fold in those with two ε4 alleles (Roses, 1996). The ε2 allele of apoE is associated with a lower risk for AD (Corder et al., 1994; Farrer et al., 1997), while the apoE ε4 allele is also associated with an earlier onset of AD (Gomez-Isla et al., 1996; Roses, 1996). This can be partly explained by the scant binding of apoE4 with Aβ which accelerates its deposition (Strittmatter et al., 1993b; Wisniewski et al., 1994), because the efficiency of complex formation follows the order of apoE2 > apoE3 ≫ apoE4

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(Tokuda et al., 2000). ApoE4 enhances Aβ aggregation and reduces Aβ clearance, that lead to promoting cerebrovascular pathology and increasing oxidative stresses (Mattson, 2004). Accumulation of aggregated $A\beta_{1-42}$ in late endosomes or secondary lysosomes may directly damage lysosomal membrane integrity (Yang et al., 1998). Furthermore, both the endosomal trafficking and colocalization with cathepsin D in cultured human brain neurons, occurs more frequently in apoE4 than apoE3 (DeKroon and Armati, 2001). Taken together, it is likely that the insertion of apoE4 into the lysosomal membrane may also facilitate neurotoxicity of Aβ peptide by destabilizing lysosomal membrane (Yuan et al., 2003).

Furthermore, apoE4 is more unstable than apoE3 and exists uniquely in 'molten globules' at low pH. Low pH in lysosomes favors the molten globular structure in apoE4 proteins which are susceptible to forming reactive intermediates. One of the distinctive properties of reactive intermediates is avid binding to phospholipid and membranes and the ability to alter and disrupt membrane structure (Dobson, 2001; Morrow et al., 2002). Although apoE4 alone cannot cause lysosomal leakage, Aβ, working in concert, facilitates release of lysosomal enzymes into the cytosol. This occurred more significantly using the arctic mutant Aβ which is prone to aggregation and fibril formation than GM6 mutant Aβ which is highly resistant to multimerization or aggregation (Ji et al., 2006). The greater propensity of apoE4 than apoE3 or apoE2 to form a molten globule has potential implications in neurodegeneration. In addition, the capacity of human apoE binding with HNE, thus, eliminating free HNE, is in the order of apoE2 > apoE3 ≫ apoE4, and this was in agreement with the preventive activity of apoE against cell death caused by HNE (Pedersen et al., 2000). Accordingly, the possession of apoE 4 with the weakest HNE elimination ability leads to accumulation of HNE in neurons.

2.3. Calpain cleavage of carbonylated Hsp70.1

Although the earliest detectable pathological change in the AD brain is at present thought to be Aβ accumulation, it is likely that Aβ accumulation is necessary but not sufficient to produce the clinical dementia. It is well known that some individuals show all of the diagnostic neuropathological evidence of AD at autopsy without expressing dementia during their life. It is also true that other individuals are relatively resistant to Aβ depositions because of protective genetic factors, environmental influences, exercise, and/or nutritional interventions (Stranahan and Mattson, 2012). Presumably, cognitive decline would occur only in the setting of Aβ depositions plus certain event (for example, synaptic loss) prone to neurodegeneration (Sperling et al., 2009). Then, what is the more essential and indispensable causative factor of neurodegeneration in AD?

Enhanced proteolysis may explain neurodegeneration in relation to ischemia/reperfusion or age-related cerebral ischemia. Widespread activation of μ-calpain is well known to occur in the AD brains, showing a 3–7-fold increase (Fig. 3d) (Saito et al., 1993; Taniguchi et al., 2001). Given the large number and wide range of physiological μ-calpain substrates, however, it is difficult to detect the in vivo substrate in the pathological conditions (Bevers and Neumar, 2008). Accordingly, much had remained unknown until recently about the precise mechanism of calpain-mediated neuronal injury and the specific calpain substrate in vivo that plays a central role in the neuronal death following ischemia/reperfusion. Intriguingly, using the monkey brains, Oikawa et al. (2009) found that carbonyl modification of Hsp70.1 is remarkably (more than 10 fold) increased in the hippocampal CA1 tissues after ischemia/reperfusion, and the carbonylation site is Arg469 (i.e. key site) of Hsp70.1 (Fig. 4a). In addition, from the in vitro experimental paradigm, Sahara and Yamashima (2010) suggested that the in vivo calapain substrate in the postischemic CA1 neurons should be 'carbonylated Hsp70.1′. They found that carbonylated Hsp70.1 in the CA1 tissue becomes much more susceptible to the calpain cleavage after artificial oxidative injuries by HNE (Fig. 4c–e) or hydrogen peroxide.

Wei et al. (1995) first reported a role for Hsp70 as an essential factor for cancer cell survival in its depletion study (Kirkegaard and Jäättelä, 2009). The cancer cell death induced by the depletion of Hsp70 was characterized by the release of cathepsins into the cytosol, and cathepsin inhibition provided a significant cytoprotection (Nylandsted et al., 2000; Nylandsted et al., 2004). Furthermore, exogenous Hsp70 effectively inhibited lysosomal destabilization induced by various stresses (Nylandsted et al., 2004; Gyrd-Hansen et al., 2006; Bivik et al., 2007). It is suggested from these data that the potent cytoprotective effect of Hsp70 is partially due to stabilization of lysosomal membranes. Both calpain activation and HNE-induced carbonylation of Hsp70.1 were identified not only in the ischemic monkey brain but also in the brain of mild cognitive impairment (MCI) (Fig. 4b) and Alzheimer patients (Sultana et al., 2010). Accordingly, the concept of 'calpain-mediated cleavage of carbonylated Hsp70.1' (Sahara and Yamashima, 2010), presumably contributes to establishing a relationship between ischemic and degenerative neuronal death (Yamashima, 2012). Here, the author would like to forward such an insight that Alzheimer neuronal death may occur by the same calpain–cathepsin cascade with ischemic neuronal death.

Interestingly, Hsp70.1 dysfunction can also explain the mechanism of neurofibrillary tangle formation (Fig. 5b). Tau is a microtubule-associated protein predominantly expressed in axons, and plays an important role in the maintenance and stabilization of microtubules. While normal tau promotes assembly and stabilizes microtubules, the non-fibrillized, abnormally hyperphosphorylated tau sequesters normal tau and disrupts microtubules. Since mutations in the tau gene cause hereditary tauopathies including Pick's disease, corticobasal degeneration, and progressive supranuclear palsy, tau dysfunction is sufficient to cause neuronal degeneration (Feany and Dickson, 1996). In AD, tau dissociates from microtubules and self-associates to form both fibrillar and prefibrillar oligomeric aggregates (Iqbal et al., 2009; Patterson et al., 2011a). Aggregation and accumulation of tau form neurofibrillary tangles which inhibit kinesin-dependent fast axonal transport. This becomes critical for neuronal function, causing cognitive decline and neuronal degeneration in AD. However, Hsp70, as a molecular chaperone, prevents protein misfolding and aggregation. Previous data (Dou et al., 2003; Petrucelli et al., 2004) suggest that Hsp70 attenuates tau toxicity by maintaining tau in a soluble, non-aggregated state and by facilitating degradation of aggregated tau species. Hsp70 interacts directly with ubiquitin ligase, carboxyl terminus of the Hsc70-interacting protein (CHIP) that has bound insoluble and hyperphosphorylated tau to regulate its ubiquitination, degradation and aggregation (Petrucelli et al., 2004; Dickey et al., 2006). Recently, Patterson et al. (2011b) demonstrated that Hsp70 directly inhibits tau aggregation by a mechanism involving preferential associations with soluble, monomeric and prefibrillar oligomeric tau species, and that Hsp70 prevents the toxic effect of preformed tau aggregates on anterograde fast axonal transport (Fig. 5b). Accordingly, Hsp70.1 disorder can cause not only lysosomal stabilization but also tau aggregation.

3. Hsp70-related molecules indispensable for the lysosomal stabilization

Lysosomes degrade a variety of macromolecules such as proteins, glycoconjugates, lipids, and nucleic acids to their building blocks (Cuervo and Dice, 1998). They provide cells with the ability

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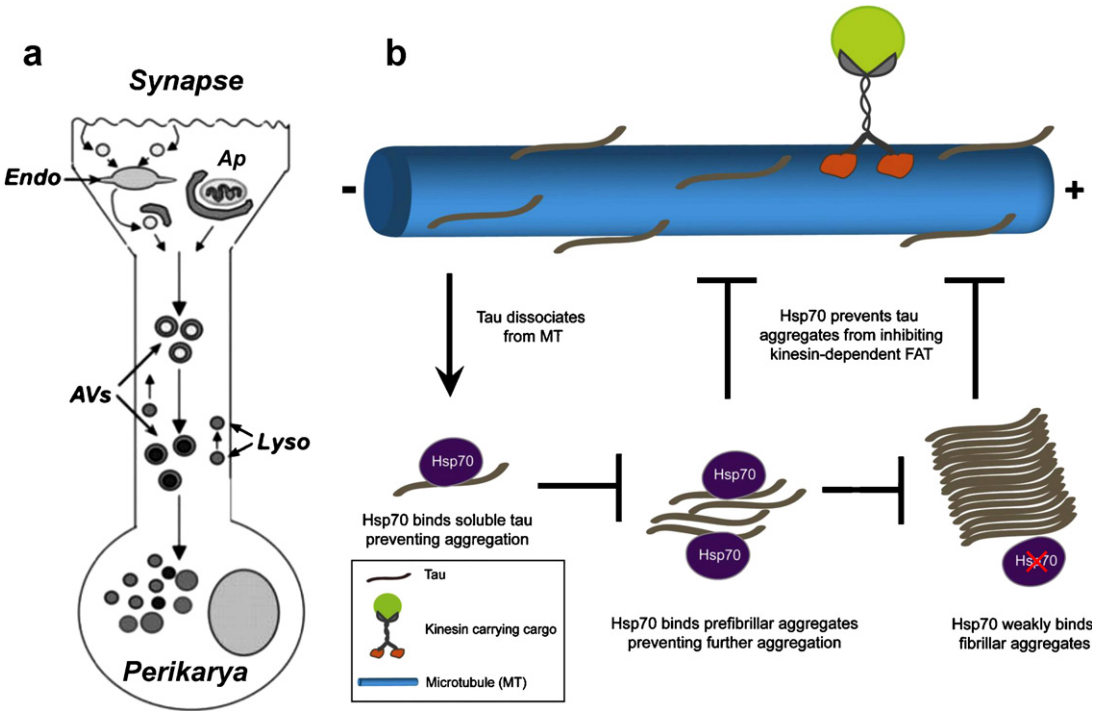

Fig. 5. Trafficking of autophagic vacuoles (AVs) along the microtuble, and its impairment by Hsp70 dysfunction. (a) Endocytosis and autophagy are highly active at synaptic terminals. For the efficient clearance of endosomes (Endo) and pre-autophagosomal membranes (Ap) being formed in synapses, nascent AVs mature to autophagolysosomes during their retrograde transport, by fusing with anterogradely transported lysosomes (Lyso). The kinesin carrying cargo (Fig. 5b) and the microtuble function like a train and railway, respectively, for such an axonal transport (cited from J Cell Sci 120, 4081–4091, 2007). (b) Model of the effects of Hsp70 dysfunction on tau aggregation. Displacement of tau from the microtubule allows for its self-association and deposition. However, molecular chaperone Hsp70 binds soluble, monomeric tau that dissociates from the microtubule, thus preventing both tau aggregates and their inhibition on kinesin-dependent fast axonal transport. Accordingly, Hsp70 dysfunction (×) which was caused by its carbonylation and cleavage (Fig. 4), leads initially to tau aggregation, and eventually to the impaired axonal transport. Simultaneously, Hsp70 dysfunction may cause neurodeneration, because impaired axonal transport disturbs maturation of autophagosomes and this leads to the destabilization of late endosomes/lysosomes (cited from Biochemistry 50, 10300–10310, 2011).

to recycle long-lived proteins and damaged organelles by autophagy. Autophagy is of special relevance also for the membrane trafficking and degradation, and another role of autophagy is that it is a source of bis(monoacylglycerol)phosphate (BMP) (also called lysobisphosphatidic acid). BMP is not present at the limiting (perimeter) lysosomal membrane, but is required for sphingolipid degradation at inner membranes of the acidic lysosomal compartments (Kolter and Sandhoff, 2010). Biosynthetically, BMP is formed during the degradation of phosphatidylglycerol and cardiolipin, presumably at the surface of intra-lysosomal vesicles (Amidon et al., 1996; Brotherus and Renkonen, 1977; Schulze et al., 2009). Phosphatidylglycerol is generated in the ER, while cardiolipin reaches the lysosome as a component of mitochondria by macroautophagy.

Since the discovery in the pig and rabbit lung by Body and Gray (1967), BMP has retained attention due to its original biochemical characteristics and its massive accumulation in lysosomal disorders. BMP is a minor lipid that accounts for only 1–2% of the total phospholipids of the mammalian tissues and cells. However, it is highly enriched in the internal membranes of late endosomes, accounting for about 15% of the phospholipids of this organelle and up to 70% of specific domains of internal membranes (Kobayashi et al., 1998, 2002). BMP plays an important role especially in the control of cellular cholesterol distribution, and also in the late endosomal/lysosomal degradative pathway. It exhibits an unusual sn1:sn1′ stereoconfiguration, based on the position of the phosphate moiety on the carbon 1 and 1′ of the glycerol backbone (Hullin-Matsuda et al., 2009). One fatty acid is attached to each of the glycerol moieties, for example, docosahexaenoic acid (DHA) and DHA (22:6n-3), stearic acid and arachidonic acid, or oleic acid and oleic acid. One characterisic of BMP is a selective incorporation of DHA, which may confer specific biophysical and functional properties. The mechanisms underlying both the high proportion of DHA in BMP and the formation of 22:6/22:6-BMP molecular species are still unknown (Hullin-Matsuda et al., 2009). However, it is likely that BMP molecules containing DHA will favor increased membrane fluidity or membrane fusion with Hsp70.1 and/or lysosomal internal membranes owing to its flexibility (Fig. 6a). However, 22:6/22:6-BMP becomes a privileged target for lipid peroxidation upon oxidant conditions (Bouvier et al., 2009). Accordingly, 22:6/22:6-BMP, being very prone to peroxidation, could exert oxidant action within its close environment, in particular toward cholesterol, then oxysterols are deleterious to the cells (Hullin-Matsuda et al., 2009).

Acid sphingomyelinase (ASM; EC 3.1.4.12) is a glycoprotein, being crucial for the pathogenesis of the rare, recessively inherited lysosomal storage disorder, Niemann–Pick disease (type A and type B). Secondary to sphingomyelin storage, many other lipids such as cholesterol and gangliosides accumulate within lysosomes, leading to a plethora of abnormalities in the cell function (Schuchman, 2010). ASM is one of a family of enzymes that catalyzes breakdown of sphingomyelin to produce ceramide by cleavage of the phosphorylcholine linkage (Fig. 6b). It is crucial for the normal membrane turnover, the integrity of lysosomes, and for the ceramide-related signaling. ASM fulfills a dual role—it has an essential housekeeping function within the lysosomes and late endosomes, whereas it has an important role for the activation of apoptotic signaling at the cell surface (Smith and Schuchman, 2008). Accordingly, it is likely that activation of ASM at the cell surface leads to apoptosis (Smith and Schuchman, 2008), whereas inactivation of ASM within lysosomes leads to necrosis (Yamashima, 2012). In internal vesicles and membranes of lysosomes,

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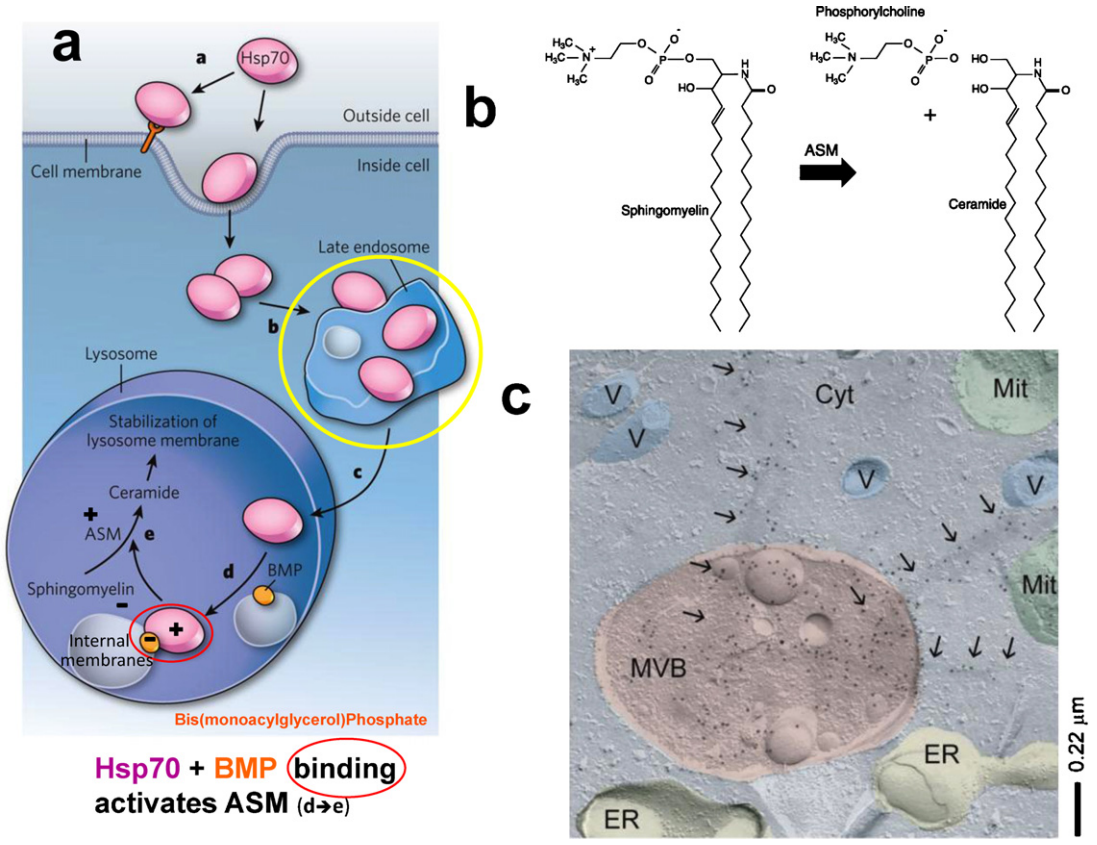

Fig. 6. The lysosomal membrane-stabilizing effect of Hsp70.1, and Aβ-induced permeabilization of the multivesicular body (MVB). (a) When recombinant Hsp70 is added to cancer cells, it is transported into the lysosome, then interacts with bis(monoacylglycero)phosphate (BMP); a membrane-bound, anionic phospholipid. The Hsp70–BMP interaction (red circle) enhances association of BMP with acid sphingomyelinase (ASM: EC3.1.4.12), which can activate this enzyme so that ASM breaks down sphingomyelin to generate ceramide. Although the exact role of ceramide remains to be determined, the increased production of ceramide by cascade **d** to **e** in lysosomes protects lysosomal membranes from destabilization/rupturing. Please, note that BMP is localized not at the lysosomal limiting membrane but at the internal membranes. Late endosome (yellow circle) is consistent with MLBs shown in Figs. 1b and c, 2c and 7b (cited from Horváth and Vígh, 2010). (b) ASM catalyzes breakdown of sphingomyelin by cleavage of the phosphorylcholine linkage, thereby producing a bioactive lipid, ceramide (cited from FASEB J 22, 3419–3431, 2008). (c) After adding freshly dissolved $A\beta_{1-40}$ peptide to the medium, the cell culture system of J774A.1 cells (murine mononuclear phagocytes) reproducibly leads to the formation of an Aβ plaque. By the transmission electron microscope observation of freeze-fractured replica, immunogold labeling (arrows) shows that internalized Aβ peptides become sorted to the MVB where amyloid fibrils grow out, thus penetrating the vesicular membrane (arrows; please, compare with Figs. 1c and 2c). Ultimately, these events lead to death of the affected cell, and intracellular Aβ peptides are released into the extracellular space to form an Aβ plaque. A similar mechanism can be applied for the AD brain. MVBs are essentially consistent with late endosomes/lysosomes. MVB: multivesicular body, V: other vesicular structures, Mit: mitochondria, ER: endoplasmic reticulum, Cyt: cytoplasm (cited from ProNAS 107, 1942–1947, 2010).

BMP remains negatively-charged even at pH 4.2. Since ASM has an isoelectric point of around 6.8, it possesses positively-charged regions in the acidic lysosomal environment. These contribute to the interaction of ASM with the anionic membrane-bound BMP (Kölzer et al., 2004; Schulze et al., 2009). After activation by binding with BMP, ASM hydrolyzes sphingomyelin to ceramide (Fig. 6a,b), and this activity depends on its recruitment to the intralysosomal vesicular membranes by BMP through its saposin domain (Jenkins et al., 2009). With the vesicle on one side and the glycan part on the other side, lysosomal proteins such as ASM and acid ceramidase are protected from the degradation by cathepsins (Schulze et al., 2009). When the ASM-BMP interaction is disturbed, the ASM displaced from its membrane-bound lipid substrate is proteolytically degraded by lysosomal proteases (Kölzer et al., 2004).

Ceramide serves as a backbone of more complex sphingolipids such as sphingomyelin and glycosylceramide. There are two metabolic pathways leading to ceramide; the de novo pathway and the salvage pathway (Grösch et al., 2012). In ER, ceramide is produced from sphinganine and acyl-CoAs (precursors and intermediates in the sphingolipid biosynthesis pathway) by the action of ceramide synthase (CerS), and transferred to the Golgi apparatus or plasma membranes (the de novo pathway). In lysosomes, sphingomyelin, the most abundant sphingolipid in the brain, is metabolized by ASM to generate phosphocholine and ceramide (the salvage pathway). There are two primary types of sphingomyelinase with distinct subcellular localization; *acid* sphingomyelinase cleaves sphingomyelin being located within lysosmes and ER (Monney et al., 1998), while *neutral* sphingomyelinase cleaves sphingomyelin at the plasma membranes (Veldman et al., 2001). CerS catalyzes the acetylation of sphingosine with an activated fatty acid to form ceramide.

Among 6 types of CerS, CerS1 and CerS2 are responsible for the production of C18 (C18:0 and C18:1) and C24 (C24:0 and C24:1) ceramides, and both appear to be the most important ceramide synthases in the CNS. CerS1 is highly expressed in most neurons and important for the normal neuronal development, whereas CerS2 is essential for the synthesis of myelin by oligodendrocytes (Grösch et al., 2012). Accordingly, changes in ceramide levels may be closely related to various neurodegenerative disorders in humans. Cutler et al. (2004) have shown in the AD brain that C18:0 and C24:0 ceramides are elevated in the middle frontal gyrus being associated with extensive Aβ plaques and tangles, and this correlates with disease progression and severity. However, since ceramide levels increase when autophagy is induced by oxidative stresses, and the levels of C18:0 and C24:0 ceramide in cultured

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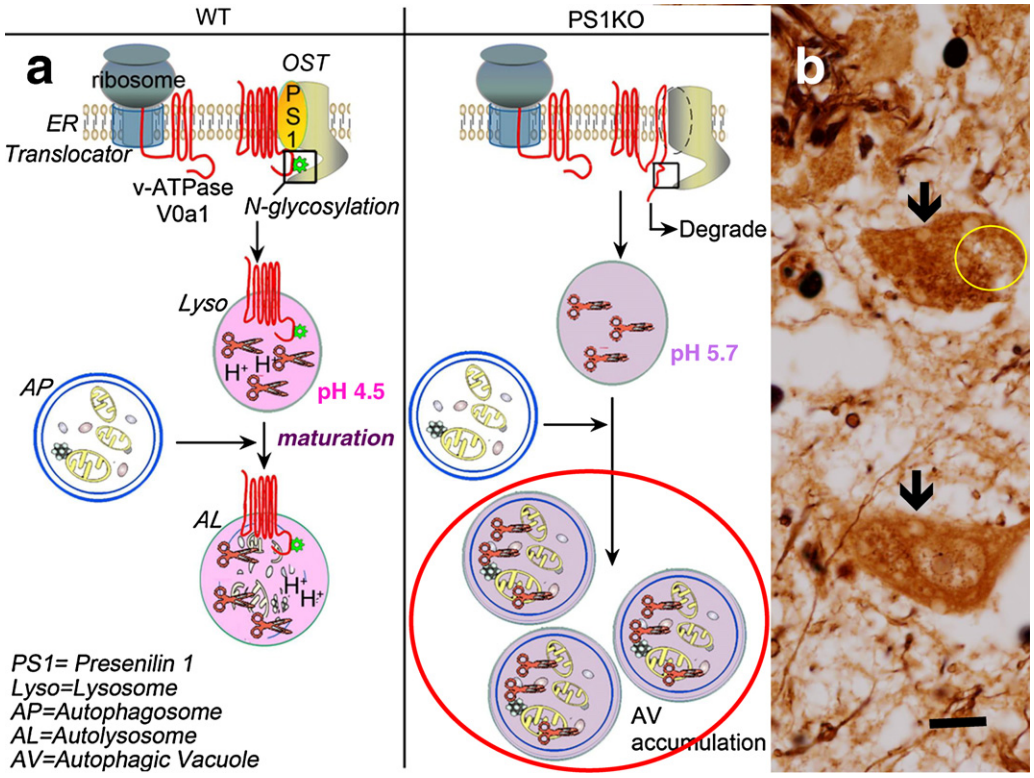

Fig. 7. Normal and impaired macroautophagy, and formation of autophagic vacuoles. (a) The AD-related protein, presenilin 1 (PS1) is essential for the lysosomal acidification and protein degradation, because v-ATPase V0a1 subunit glycosylation and trafficking to lysosomes requires PS1. In normal cells (WT), PS1 mediates the post-translational addition of an N-glycan (rectangle) to the immature V0a1 subunit through interaction with the oligosaccharyltransferase (OST) complex and the translocon. Then, v-ATPase contributes to sufficient acidification (pH 4.5) of lysosomes, and autophagosomes (AP) fuse with acidified lysosomes (Lyso) (called 'maturation') to promote degradation of the autophagosome's contents within autolysosomes (AL). In contrast, neurons in PS1-knock-out mice (PS1KO) show glycosylation impairment in ER and lysosomal acidification deficits (pH 5.7), thereby interfering with the subsequent autophagosome clearance during macroautophagy, and causing virtually complete failure of macroautophagy and accumulation of autophagic vacuoles (AV being consistent with MVBs, red circle) (cited from Cell, 141, 1146–1158, 2010). (b) In AD neurons associated with impaired lysosomal proteolysis, impairment of autophagy leads to massive accumulation of autophagic vacuoles called granulo-vacuolar degenerations (black arrows) which means accumulation of incompletely degraded proteins in autolysosomes. Some autophagic vacuoles comprise of aggregates of microvesicles (yellow circle) as shown also by electron microscopy (Fig. 2c). Robust autophagic vacuole accumulation precedes neuron death.

hippocampal neurons increased in response to Aβ (Cutler et al., 2004), it is not clear whether the above elevation is a consequence of Aβ elevation or the cause of AD.

Incorporation of ceramide into membrane can increase its rigidity by ordering the acyl chains, dividing the membrane into microdomains (rafts/caveolae), promoting membrane permeability and channel formation, facilitating membrane fusion and inducing fission, budding and vesicle formation (Grösch et al., 2012). For instance, ceramide protects the lysosomal membrane from rupturing (Fig. 6a) (Kirkegaard et al., 2010; Petersen et al., 2010; Petersen and Kirkegaard, 2010), because the increased concentration of lysosomal ceramide possibly facilitates fusion of lysosomes with other intracellular vesicles and cell membranes (Heinrich et al., 2000). Since generation of ceramide greatly alters the membrane properties, increase in lysosomal ceramide content might in itself have a positive impact on the lysosomal membrane stability (Petersen et al., 2010; Petersen and Kirkegaard, 2010). In addition to its structural role in the lysosomal membrane, ceramide acts as a second messenger in various signaling pathways at the plasma membranes. Ceramide can promote differentiation, proliferation, death or survival (Billis et al., 1998; Andrieu-Abadie et al., 2001), depending on cell type, mode (de novo or catabolic reaction), subcellular location, and its chain length (Diringer et al., 1972; Cutler et al., 2004). One should note that all ceramides are not created equal, and ceramides likely exert distinct effects in different cellular/subcellular compartments by virtue of access to distinct molecules (Jenkins et al., 2009). The exact role of ceramide in neuronal survival or death still remains controversial and should be elucidated further.

4. Impaired autophagy due to cleavage of lysosomal guardian Hsp70.1

4.1. Autophagy failure leading to autophagic vacuoles

Proteolysis has been an active area of AD research over the past two decades. In neurons, two proteolytic systems are mainly responsible for the turnover of proteins and organelles: one is the ubiquitin-proteasome system while another is the autophagic-lysosomal systems (Goldberg, 2003; Ihara et al., 2012). The former is responsible for degrading soluble proteins (mainly those with short half-lives), whereas the latter is primarily responsible for clearing insoluble protein aggregates and cellular organelles (mainly those with long half-lives). For the postmitotic cell, neurons, which cannot dilute toxic protein build-up by cell division, the autophagic-lysosomal system is indispensable to sort and clear damaged proteins and facilitate vital signaling functions. Especially for the aged neurons, their survival depends on eliminating a growing burden of long-lived, oxidized, aggregated, or potentially toxic proteins and degrading, dysfunctional, unwanted organelles.

Two pathways of endocytic (Fig. 6a) and autophagic (Fig. 7a) share in common the role of delivering unneeded cellular materials to lysosomes for recycling to provide new synthesis and energy.

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Endocytosis supports unique neuronal functions, including synaptic transmission and plasticity that proceed in synaptic terminals at great distances from the perikaryon. Endosome abnormalities, being closely related to the synaptic loss and memory disorders, are the earliest specific events in the AD brain. Neuronal endosome enlargement, specific for AD, develops in pyramidal neurons of the neocortex at a stage when plaques and tangles are restricted only to the hippocampus (Cataldo et al., 2000; Ihara et al., 2012). Autophagy is lysosome-mediated catabolic mechanism, which plays an important role to maintain cellular homeostasis by continually recycling cellular components for the biosynthesis and energy production. It is defined as the lysosomal digestion of a cell's own material such as damaged cytoplasmic components and organelles. For example, lower eukaryotic organisms such as yeast can survive nutrient starvation conditions by degrading non-essential cell constituents to utilize as an energy source. The same may occur in the mammalian cells when facing the severe stresses such as nutrient deprivation, hypoxia, heat, irradiation, etc.

Autophagy is classified into three processes by which intracellular constituents reach the lumen of lysosomes for degradation: chaperone-mediated autophagy, microautophagy, and macroautophagy (Cuervo, 2004; Mizushima et al., 2008; Levine et al., 2011). All autophagic vacuoles eventually fuse with lysosomes which provide hydrolases as degradating enzymes. In chaperone-mediated autophagy, cytosolic proteins such as APP and synuclein containing a KFERQ motif are selectively targeted to the lysosomal lumen for degradation. For example, the processing of tau occurs partially by chaperone-mediated autophagy. Upon association of tau with the lysosomal limiting membrane via heat-shock constitutive protein 70 (Hsc70) and lysosome-associated membrane protein-2a (lamp-2a), tau is translocated into the lumen of lysosomes thereby allowing proteolysis by cathepsins (Wang et al., 2009; 2010). In microautophagy the lysosome invaginates its own membrane to uptake segments of the cytoplasm (Massey et al., 2004; Nixon, 2006; Kragh et al., 2012). Macroautophagy, being conserved from yeast to mammals, is the principal degradative pathway necessary for the aged or oxidatively-damaged organelles and long-lived proteins. It is primarily a protective process for neurons, and impaired clearance of autophagic vacuoles by macroautophagy may be closely related to neurodegeneration in AD (Cardoso et al., 2010).

In macroautophagy, regional sequestration of cytoplasm within an enveloping double membrane structure (pre-autophagosomal membranes), creates vacuoles designated autophagosomes that are continuously generated at the distal ends (synapses) of axons or dendrites (Fig. 5a) (De Duve and Wattiaux, 1966; Klionsky et al., 2008). Newly formed autophagosomes are normally eliminated efficiently in neurons by fusing with lysosomes or late endosomes to form autolysosomes, because such fusion being called 'maturation' can provide hydrolases necessary for degaradation (Boland et al., 2008; Noda et al., 2009). Then, both lysis of the autophagosome inner membrane and breakdown of the contents occur within autolysosomes (Fig. 7a) (Mizushima et al., 2008). For achieving this, autophagosomes must travel retrogradely long distances before being efficiently degraded by fusing with lysosomes which usually concentrate in or near the perikaryon (Fig. 5a) (Nixon et al., 2005; Nixon, 2007). Accordingly, intact microtubules are needed (Fig. 5b) for the trafficking of endosomes or autophagosomes, which facilitates their fusion with lysosomes at the destination (Kovács et al., 1982; Köchl et al., 2006). Such autophagic pathway is constitutively active and highly efficient in neurons, and is essential for the neuronal survival. Since cytosolic tau and proteolytic products can be degraded by macroautophagy (Dolan and Johnson, 2010; Wang et al., 2010), its impairment results in accumulation and further aggregation of tau (Hamano et al., 2008). Accordingly, dysfunction of tau proteolysis in AD neurons conceivably leads to accumulation of tau fragments that are prone to aggregation (van Echten-Deckert and Walter, 2012).

Multivesicular bodies (MVBs), showing fingerprint-like structures, are vesicular organelles morphologically defined by a limiting membrane and characteristic inner vesicles at the ultrastructural level (Figs. 1b and c, 2c and 8b: yellow circles), and considered a sorting compartment within the endocytic pathway (Gruenberg, 2001). Both early endosomes and, more typically, late endosomes (Fig. 6a: yellow circles) have been described as MVBs (Gruenberg and Maxfield, 1995; Katzmann et al., 2001). When early endosomes mature into late endosomes, regions of the surface membrane bud off into the endosome lumen to form more MVBs (van der Goot and Gruenberg, 2006). MVBs are involved in the regulated trafficking of several proteins and membrane receptors (Gruenberg and Stenmark, 2004). For example, after endocytosis of APP from the plasma membrane (Koo and Squazzo, 1994), internalized APP traffics from the plasma membrane to MVBs (Fig. 6c) (Yamazaki et al., 1996; Friedrich et al., 2010). Then, Aβ is generated via consecutive cleavage by β-Site APP cleaving enzyme (BACE) (Vassar et al., 1999) at endosomal vesicles (Huse et al., 2000) and γ-secretase in late endosomes (Vetrivel et al., 2005).

MVBs can be identified not only in the normal neurons but also in the AD neurons (Takahashi et al., 2002). Mounting evidence shows that failure of the substrate proteolysis within autolysosomes causes increased formation and accumulation of autophagic vacuoles (Figs. 1b and c, 2c and 7a and b) (Nixon et al., 2005; Nixon, 2006, 2007; Nixon and Yang, 2011). At the electron microscopic observation autophagic vacuoles often look like MVBs, because lipid components are more efficiently preserved by the glutaraldehyde and osmium fixation compared to the formalin fixation for light microscopy (Figs. 1b and c and 2c: yellow circles). In both APP mutant transgenic mice and human AD brains, progressive accumulation of $A\beta_{42}$ occurs in neuronal MVBs (Takahashi et al., 2002), and Aβ accumulation impairs multivesicular body sorting by inhibiting the ubiquitin-proteasome system (Almeida et al., 2006). Then, the ordered MVB function is disturbed and some Aβ fibrils grow out, thus penetrating the vesicular membrane (Fig. 6c, arrows) (Friedrich et al., 2010). When degenerating neurons die ultimately, intracellular amyloid peptides are released into the extracellular space to aggregate each other, consequently forming senile plaques (Fig. 1a). Accordingly, extracellular Aβ plaques contain a spectrum of proteins that were originally intracellular. For instance, Aβ plaques commonly contain lysosomal proteases like cathepsin D or molecular chaperones like Hsp70 or Hsp20 (Cataldo and Nixon, 1990; Roher et al., 1993; Wilhelmus et al., 2007). Since $A\beta_{42}$ accumulates in MVBs within presynaptic and especially postsynaptic compartments (Takahashi et al., 2002; Langui et al., 2004), Alzheimer transgenic mice show impaired synaptic plasticity and learning, as Aβ levels rise, months before the appearance of Aβ plaques (Chapman et al., 1999).

During clearance of autophagosomes, fusion with lysosomes introduces more than 60 hydrolases into the autophagosomes. A membrane-spanning proton pump, v-type $[H^+]$-ATPase (v-ATPase), lowers the intralumenal pH to the level optimal for the protease activation (Sun-Wada et al., 2003). However, lysosomal destabilization would affect downstream autolysosome formation and abolish supply of metabolic substrates provided by autophagy (Repnik and Turk, 2010). Fusion with the destabilized lysosomes may disturb clearance of autolysosomes, consequently autophagic vacuoles are formed and accumulate (Fig. 7a). In *Caenorhabditis elegans*, autophagy synergizes with lysosomal proteolytic pathways to facilitate necrosis, and excessive autophagosome formation is induced early during necrotic cell death (Samara et al., 2008). Similar situations can be observed in programmed neuronal necrosis in AD; for example, autophagic dense vesicles (Fig. 1b) or

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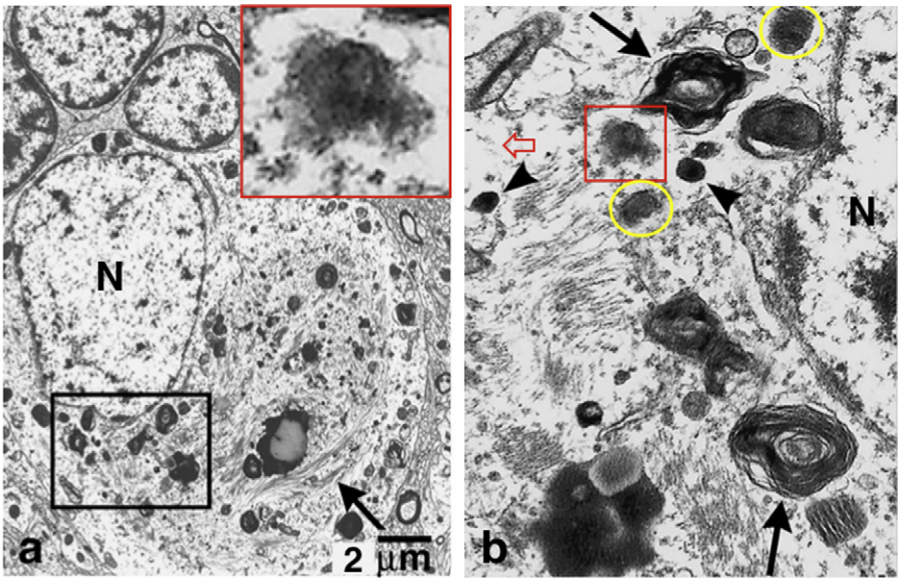

Fig. 8. Electron microscopic photographs showing impairments of lysosomal integrity and maturation in the degenerating AD neuron. In affected neurons of AD, newly formed autophagosomes are not efficiently cleared by fusion with lysosomes. Accordingly, the perikarya of the degenerating neuron containing neurofibrillary tangle (a; long arrow) is filled with autophagic vacuoles containing multilamellar bodies (b; long arrows). It is conceivable that multilamellar bodies derive from the endocytosis of plasma membranes while non-lamellar lysosomes derive from autophagy of non-membranous organelles. The degenerating neuron shows a remarkable decrease of mitochondria, ER and free ribosomes. There are three types of lysosomes; one with an electron-dense core being surrounded by the distinct limiting membrane (b: arrowheads), another with double-contours (a, b: red rectangles), and the other containing many vesicles being reminiscent of MVBs (b: yellow circles). Double contour of the lysosome (a, b: red rectangles) appears to have lost its delimiting membrane due to the destabilization and/or partial rupture, and shows a contrast with the vivid lysosomes (b, arrowheads). Black rectangle in a is magnified in b, while red rectangle in b is magnified in a. N: nucleus (cited from J Neuropathol Exp Neurol 64, 113–122, 2005).

autophagic vacuoles (Figs. 1c and 7b) accumulate in dystrophic neurites or perikarya of degenerating neurons, respectively (Nixon et al., 2005), probably due to impairment of autophagosome maturation into autolysosomes (Fig. 7a) (Yu et al., 2005). However, the molecular basis for the autophagosome clearance by lysosomes (i.e. maturation) and/or the profuse accumulation of autophagic vacuoles in the AD-affected neurons has not been clarified completely. Accordingly, until recently one could not conclude whether the autophagy per se constitutes an independent pathway of programmed neuronal death or merely one of the downstream manifestations of a much more complex process (Yuan et al., 2003). Even now, no one can conclude whether impaired clearance of autolysosomes and/or accumulation of autophagic vacuoles are contributing to neuronal death or protecting against it. Interestingly, autophagosomes were found to accumulate relatively rapidly when their clearance was prevented by blocking lysosomal degradation with cathepsin inhibitors in primary cortical neurons (Boland et al., 2008). Defective clearance, i.e. accumulation of autophagosomes associated with the lysosomal depletion was confirmed in the degenerating dopaminergic neurons of the Parkinson's disese patients and its animal models (Dehay et al., 2010). Furthermore, studies on various lysosomal storage disorders revealed evidence of impaired clearance of autophagic vacuoles (Kiselyov et al., 2007; Settembre et al., 2008). Taken together, it is most probable that accumulation of autophagic vacuoles via lysosomal destabilization leads to neuronal death.

4.2. Congenital or acquired mechanisms of autophagy failure

Mutations in the presenilin genes are known to be the common cause of familial AD. Presenilin is the catalytic subunit of the γ-secretase activity that liberates Aβ peptide from the C-terminus of APP (Wakabayashi and De Strooper, 2008). The pathogenic effects of presenilin 1 mutations in AD are commonly ascribed to increased generation of the neurotoxic Aβ peptide from APP. Furthermore, presenilin 1 is crucial for the subcellular membrane trafficking (Esselens et al., 2004; Wilson et al., 2004; Sannerud and Annaert, 2009), and is required for the lysosomal turnover of autophagic and endocytic protein substrates. Disruption of the autophagy pathway promotes depositions of amyloid plaques (Pickford et al., 2008; Tooze and Schiavo, 2008). In addition, more importantly, presenilin 1 is essential for targeting of v-ATPase V0a1 subunit to lysosomes to maintain lysosomal acidification necessary for the proteolysis during autophagy (Fig. 7a) (Yoshimori et al., 1991; Lee et al., 2010). Presenillin 1 mutations cause impaired autophagy as a result of a selective impairment of autolysosome acidification and cathepsin activation, which may affect neuronal survival (Lee et al., 2010).

In the familial AD, mutant presenilin 1 disrupts autophagy by impeding lysosomal acidification (Fig. 7a). Then, such a question emerges how autophagy failure occurs in the majority of AD cases who are free from presenillin 1 mutations. The author speculates that impaired autophagy in AD also arises from impaired clearance of autophagosomes due to the age-related Hsp70.1 dysfunction. The latter induces both (1) the failure of maturation process (i.e. trafficking of autophagosomes in the synaptic and dendritic area to lysosomes in the perikarya) (Fig. 5a), and the resultant (2) autolysosomal proteolytic failure by its inappropriate acidification that leads to autophagic vacuole accumulation (Fig. 7a). It is noteworthy that both failure can occur by the carbonylation and cleavage of key molecule 'Hsp70.1′ (Fig. 4) in the sporadic AD patients who are free from presenillin 1 mutations. In the most common, sporadic AD, impaired autophagy can be explained by calpain-mediated cleavage of carbonylated Hsp70.1 that causes ASM dysfunction (Fig. 4 c,d,e, 6a) (Yamashima, 2012). The binding of normal Hsp70.1 with BMP regulates the enzyme activity of ASM which generates ceramide for the lysosomal membrane stabilization. Accordingly, it is probable in the Alzheimer brain that HNE-induced carbonylation of Hsp70.1 and calpain-mediated cleavage of the carbonylated Hsp70.1 (Sahara and Yamashima, 2010), might cause down-regulation of BMP and ASM activities, which leads to storage of sphingomyelin with the resultant lysosomal dysfunction

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and rupture (Fig. 8). Hsp 70.1 dysfunction causes not only autophagy failure but also defective microtubular trafficking by inhibiting kinesin-dependent fast axonal transport (Fig. 5b) (Patterson et al., 2011b).

Multilamellar bodies (or myeloid bodies) are ultrastructurally composed of concentric membrane layers with an electron-dense core (Fig. 8b), but reveal as autophagic vacuoles on light miscroscopy (Fig. 7b). Lysosomal nature of them has been demonstrated by the presence of various lysosomal enzymes (Hariri et al., 2000), and they form during the endocytic pathway of plasma membranes (phosphilipid-like sphingomyelin) under both physiological and pathological conditions. For example, multilamellar bodies are responsible for the surfactant secretion in type II alveolar cells to prevent alveolae from collapse during respiration but also accumulate in other cell types under pathological conditions such as lysosomal storage diseases (Lajoie et al., 2005). As neuronal survival requires continuous lysosomal recycling or turnover of cellular constituents, lysosomal destabilization causes severe lipidosis, i.e. accumulations of multilamellar bodies (Fig. 8) (Yamashima, 2012). Macroautophagy is sensitive to the phosphatidylinositol 3 (PI 3)-kinase inhibitor, 3-methyladenine (Seglen and Gordon, 1982; Blommaart et al., 1997). Serum starvation stimulates macroautophagy in various cell lines (Munafó and Colombo, 2001; Susan and Dunn, 2001), then such stimulation of macroautophagy is associated with increased size and concentric lamellar morphology of multilamellar bodies (Hariri et al., 2000; Lajoie et al., 2005). The formation of multilamellar bodies starts with inward budding of the limiting endosomal membrane and requires the sequential action of three endosomal sorting complexes required for transport (ESCRT-I, -II, -III) (Hopkins et al., 1990). They are found in numerous cell types where they function in lipid storage and secretion (Schmitz and Müller, 1991).

Drug-induced phospholipidosis is an artificial phospholipid storage disorder that is characterized by the excessive accumulation of multilamellar phospholipids in tissues (Tengstrand et al., 2010). For example, excess intake of drugs such as tetracyclines and anti-histamines cause phospholipidosis in the renal cortex. Similarly, numerous multilamellar bodies can be detected in the urinary sediment of patients with acute renal failure receiving gentamycin. The accumulation of undigested drug-phospholipid complexes and an alteration in lysosomal enzyme activity results in the intracellular accumulation of multilamellar bodies. It may have an adaptive or detoxification effect by sequestering and eliminating drugs and their metabolites from the cell (Tengstrand et al., 2010). Putative b1–6 branching and polylactosamine glycosylation of multilamellar body glycoproteins might enhance their resistance to degradation by lysosomal proteases or modify interactions between the lamellar components, thereby favoring lamella formation (Hariri et al., 2000). Initially, single or multiple foci of lamella appear within an autophagic vacuole, which transform into multilamellar structures. Treatment of the lung type II alveolar cells with leupeptin, an inhibitor of many lysosomal proteases, results in the progressive transformation of the multilamellar bodies into electron-dense autophagic vacuoles and eventual disappearance of these bodies. In contrast, treatment with 3-methyladenine, an inhibitor of autophagic sequestration, results in the significantly reduced expression of multilamellar bodies and the accumulation of inclusion bodies resembling nascent or immature autophagic vacuoles (Hariri et al., 2000). These data indicate that the selective impairment of lysosomal degradation is indispensable for the formation of multilamellar bodies.

Intriguingly, autophagic vacuoles containing multilamellar bodies can be seen in various situations such as congenital and drug-induced lysosomal storage diseases (Lajoie et al., 2005; Tengstrand et al., 2010), Alzheimer disease (Nixon et al., 2005), Niemann-Pick disease (Macauley et al., 2008; Saunders and Wenger, 2008), and cerebral ischemia (Yamashima, 2012). Accordingly, it is likely that not only congenital impairment of the lysosomal acidification but also acquired disorders of lysosomal factors including Hsp70.1, BMP, ASM, etc. would cause formation of autophagic vacuoles (Figs. 1, 2c, 7b) and/or multilamellar bodies (Fig. 8) as a result of impaired autophagy and lysosomal lipidosis in AD neurons.

5. Evidence of lysosomal rupture in vitro and in vivo

Brunk and his colleagues established the concept of lysosomal membrane permeabilization (LMP) in a series of works using cultured cells undergoing artificial oxidative stresses (Brunk et al., 1997; Brunk and Svensson, 1999; Li et al., 2000; Antunes et al., 2001; Kågedal et al., 2001). Taken together with the monkey data of the author's group (Yamashima et al., 1996, 1998), it became clear that low levels of stress cause LMP and apoptosis, whereas high levels of stress cause lysosomal rupture and necrosis (Bursch, 2001; Nylandsted et al., 2004; Kirkegaard and Jäättelä, 2009; Turk and Turk, 2009). LMP was suggested to induce apoptosis by mitochondrial transmembrane potential loss or caspase activation. In contrast, lysosomal rupture can induce necrotic cell death due to extensive leakage of cathepsin enzymes (Tardy et al., 2006). There are cross-talks among cellular organelles (Ferri and Kroemer, 2001), especially between lysosomes and mitochondria (Repnik and Turk, 2010; Repnik et al., 2012), as well as between apoptosis and necrosis cascades (Yamashima, 2000).

Lysosomal cathepsins B and L as well as cathepsin D (Fig. 9a) released into the cytoplasm may damage cellular constitutive proteins and cytoskeletons. Simultaneously, they damage the lysosomal membrane from outside, or activate phospholipases that degrade all types of cellular membranes. Furthermore, they attack mitochondria to cause release of cytochrome c and other pro-apoptotic factors from outer membranes, and also to induce more H_2O_2 production by interfering with the mitochondrial electron-transporting complexes (Terman et al., 2006). The hypothesis that lysosomal leakage of cathepsin B into the cytoplasm causes apoptotic cell death would be even more persuasive, if considering data of cathepsin B knockout mice and inhibitor experiments. For example, using cathepsin B knockout mice, Guicciardi et al. (2000) showed that cathepsin B is critical to TNF-α-mediated hepatocyte apoptosis, while Houseweart et al. (2003) showed that it is the principle cause of apoptotic cell death in an epileptic animal model. Furthermore, Foghsgaard et al. (2001) showed that cathepsin B is the dominant execution protease in tumor cell apoptosis, using cathepsin B inhibitors in fibrosarcoma cells. Luo et al. (2010) also showed that the cathepsin B inhibitor CA-074Me reduced the proapoptotic biomarkers such as Bax, reduced neuronal cell death, and improved memory deficits in a traumatic brain injury model. In addition, both cathepsin D and cathepsin L being released from lysosomes, can induce the proteolytic activation of Bid, and this generates a truncated Bid that causes release of cytochrome c from mitochondria and activation of caspases-9 and caspase-3 (Stoka et al., 2001; Heinrich et al., 2004).

Until now, intriguingly, very little is known about the exact role of Aβ in the development of Alzheimer neuronal death. Yang et al. (1998) demonstrated in the neuroblastoma SH-SY5Y cells that failure of degrading aggregated $A\beta_{1-42}$ in the endosomal/lysosomal compartment causes loss of lysosomal membrane integrity (Fig. 9b-e). Actually, the cultured SH-SY5Y cells can internalize soluble $A\beta_{1-42}$ from the culture medium, which accumulate inside the endosomal/lysosomal system. However, the intracellular $A\beta_{1-42}$ is resistant to protease degradation and

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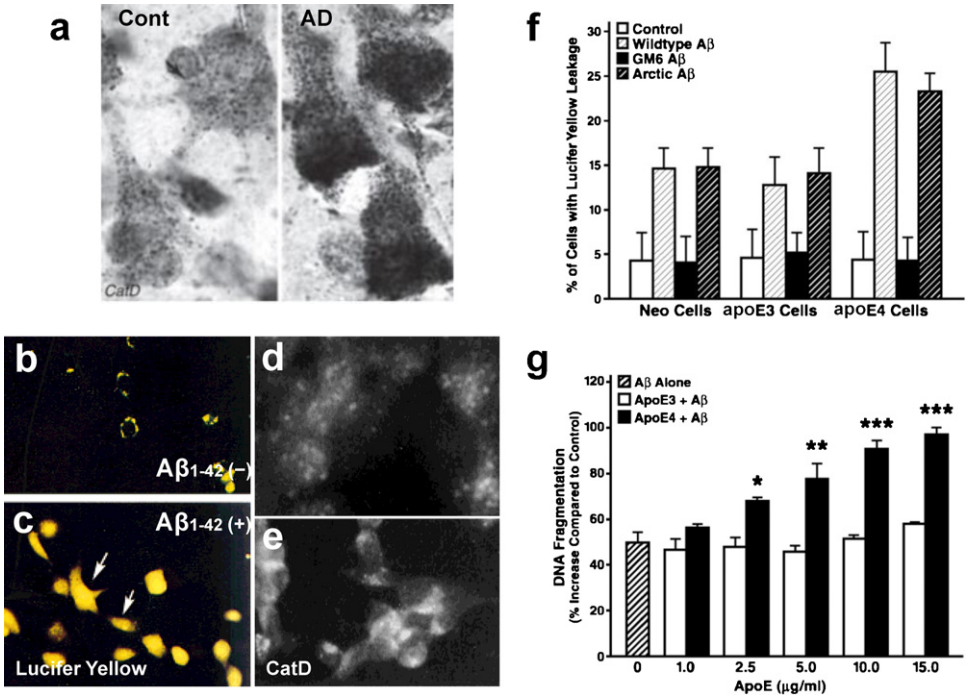

Fig. 9. In vivo and in vitro evidence of the lysosomal destabilization, and synergic action of $A\beta_{1-42}$ and apoE4 for the lysosomal destabilization and DNA fragmentation. (a) The cytoplasmic cathepsin D (CatD) immunoreactivity is more intense in AD neurons, compared to the age-matched controls (Cont). This indicates extra-lysosomal release of the cathepsin D enzymes, presumably due to the lysosomal destabilization as shown in Fig. 8 (red rectangles) (cited from Cold Spring Harb Perspect Med 2, a006361). (b and c) Lucifer yellow is the fluid-phase endocytic tracer dye. When SH-SY5Y cells are loaded with this vital dye, it is terminally trafficked to the lysosomes. In the control cells without $A\beta_{1-42}$, lucifer yellow-positive lysosomes are distributed in a punctate, perinuclear fashion (b). After incubation with $A\beta_{1-42}$, the lysosomal membranes synchronously begin to leak, then lucifer yellow shows a diffuse distribution in the cytoplasm (c). (d and e) Cathepsin D (CatD) is also abnormally redistributed in $A\beta_{1-42}$-treated SH-SY5Y cells. In the control cells, cathepsin D displays typical, perinuclear, granular endosomal/lysosomal immunostaining (d). In contrast, $A\beta_{1-42}$-treated cells display both cytoplasmic and granular immunostaining, which indicates lysosomal destabilization by $A\beta_{1-42}$ (e) (cited from J Neurosci Res 52, 691–698, 1998). (f) Comparison of Aβ-induced lucifer yellow leakage among three types of Neuro-2a cells; Neo-, apoE3-, and apoE4-secreting cells. The largest leakage occurs in apoE4 cells when incubated with wild type Aβ or Arctic Aβ. However, there were no difference of increment in response to the more soluble GM6 Aβ. Control cells; incubated without $A\beta_{1-42}$, Wild type Aβ: incubated with $A\beta_{1-42}$, GM6 $A\beta_{1-42}$; incubated with the GM6 mutated (F690S/L705P) Aβ being engineered to resist aggregation, Arctic Aβ; incubated with 'Arctic' mutated (E693G) Aβ enhancing aggregation in vitro. (g) Effect of increasing concentrations of exogenous human apoE (0–15.0 μg/ml) with Aβ on DNA fragmentation of Neo-transfected Neuro-2a cells. When compared with apoE3 (white bar), apoE4 (black bar) significantly provoked $A\beta_{1-42}$-induced DNA fragmentation in proportion to the apoE4 concentrations. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus Aβ alone without exogenous apoE4 (cited from J Biol Chem 281, 2683–2692, 2006).

induces the rapid free radical generation within lysosomes, disruption of lysosomal membrane proton gradient, and eventually lysosomal rupture. The latter provokes leakage of cathepsin D or lucifer yellow (Fig. 9e,f) into the cytoplasmic compartment. As treatment of cells with antioxidants partially blocks the release of lysosomal contents, the disruption of lysosomal membrane integrity may be caused by oxidative damage to the lysosomal membrane lipids rather than by the direct solubilization of lysosomal membrane by Aβ (Ditaranto et al., 2001). It is probable that Aβ can cause Alzheimer neuronal death not by direct effects upon lysosomal membranes but by indirect effects down-regulating lysosomal factors.

In the AD brain, microglia play a central role in mediating chronic inflammatory conditions by releasing numerous acute phase proteins such as α-antichymotrypsin, α-antitrypsin, complement proteins, and proinflammatory cytokines such as interleukins 1α, 1β, and 6, tumor necrosis factor (TNF)-α, etc. (Klegeris et al., 1997; McDonald et al., 1997; Licastro et al., 1998; Yates et al., 2000; Veerhuis et al., 2003). Because activated microglia are intimately related to senile plaques, and NSAID show protective effects (Rich et al., 1995; McGeer et al., 1996; Stewart et al., 1997; In t'Veld et al., 2001; Mackenzie and Munoz, 1998, 2001; Zandi et al., 2002), chronic inflammation has been thought to play an important role in AD pathogenesis. Besides fibrillar $A\beta_{1-42}$, soluble $A\beta_{1-42}$ also appears to be a potent activator of microglia. Using a functional genomics approach to find crucial molecular mediators, Gan et al. (2004) found 554 upregulated genes being transcriptionally induced by $A\beta_{1-42}$, and identified cathepsin B as one of the key players in the neurotoxic effects that were caused by freshly sonicated $A\beta_{1-42}$-activated microglial BV2 cells. Freshly sonicated $A\beta_{1-42}$ did not cause neuronal death when added directly to primary cultured neurons, but activated BV2 cells releasing cathepsin B caused remarkable neuronal death. Not only small interfering RNA-mediated silencing of the cathepsin B gene but also cathepsin B inhibitor CA-074 diminished the activated microglia-mediated neurotoxicity. Accordingly, cathepsin B released from the activated microglia into the medium was also thought to play an essential role in neuronal death mediated by Aβ-activated inflammatory response. Regardless of the cellular origin of cathepsin B, extra-lysosomal release of this protease has a crucial role in the development of Alzheimer neuronal death.

Lysosomal or endosomal abnormalities were thought to be one of the causative factors of AD (Cataldo and Nixon, 1990; Nixon et al., 1992; Cataldo et al., 1995). In the normal brain, most Aβ being formed during autophagy is degraded properly within lysosomes. On the contrary, in the Alzheimer brain, Aβ initially accumulates within lysosomes and late endosomes, and is subsequently trafficked into the large pool of autophagic vacuoles that becomes a major intracellular reservoir of the toxic peptide (Glabe, 2001; Yu et al., 2005). Intracellular amyloid induces generation of ROS and lipid peroxidation products. Aβ within lysosomal compartments can trigger release of hydrolytic enzymes into the cytoplasm, which causes cell death (Fig. 9) (Yang et al., 1998; D'Andrea et al., 2001; Ditaranto et al., 2001; Glabe, 2001;

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Ji et al., 2002; Takahashi et al., 2004; Friedrich et al., 2010). The neuron may ultimately lyse or die and release the insoluble Aβ into the extracellular space, where the cell debris and Aβ may serve as a nidus for the senile plaques (Fig. 1a).

Despite the numerous in vitro studies, the molecular mechanisms of lysosomal membrane disruption have been poorly understood in vivo until the '*calpain–cathepsin hypothesis*' was formulated from the ischemic monkey experimental paradigms (Yamashima et al., 1998). Owing to the formulation of this hypothesis, the cascade leading to lysosomal rupture and its effects on ischemic neuronal death have become more clear, but implication of lysosomal rupture in vivo still remains unclear in the Alzheimer neuronal death. Because of the tininess of lysosomes, it is usually difficult to observe disruption of their limiting membrane especially in the postmortem brain tissues. Accordingly, for the assessment of lysosomal membrane integrity in AD neurons by electron microscopy, one must be most careful not to underestimate presence of LMP and/or lysosomal rupture. At the careful observation, for instance, double contour of the lysosome appears to have lost its delimiting membrane (Fig. 8: red rectangles), showing a remarkable contrast with the normal lysosome, and is reminiscent of permeabilization and/or rupture of the limiting membrane. In the CA1 neurons after ischemia/reperfusion, Yamashima et al. (1996, 1998) discovered direct evidence of lysosomal membrane rupture by electron microscopy. Simultaneously, immunohistochemical analysis showed that cathepsins B and L were redistributed from lysosomes into the cytosol and/or the neuropil. Similarly, in the neurons of human AD patients, cathepsin D was redistributed from lysosomes into the cytosol (Fig. 9a). As the extent of lysosomal destabilization is distinct between ischemic neuronal death and Alzheimer neuronal death, programmed neuronal necrosis presumably occurs within days by the lysosomal rupture in acute ischemia while within years by the lysosomal permeabilization in AD.

In the AD brain, cathepsin D immunoreactivity was often localized extracellularly to the senile plaques (Cataldo and Nixon, 1990; Cataldo et al., 1995, 1996). In the rat brain, cathepsin D showed an age-dependent significant increase (Bi et al., 2000, 2003), while cathepsin L showed an age-related remarkable reduction (Nakanishi et al., 1994). A similar increase in the cathepsin D immunoreactivity associated with aging was found in neurons of dogs (Lynch and Bi, 2003). Cathepsin D is also common in the aged human brains, and shows a remarkable increase not only in the vulnerable neurons of the AD brain in advance of the onset of overt pathology (Cataldo et al., 1991; Troncoso et al., 1998; Nixon et al., 2000) but also in the cerebrospinal fluid of AD patients (Schwagerl et al., 1995). As cathepsin D can perform a limited proteolysis of a substrate even at physiological pH in the extracellular medium (Lkhider et al., 2004), cathepsin D should be active *in vivo* not only at very acidic pH but also at neutral pH. Implication of cathepsin D for Alzheimer neuronal death is reasonable, if considering the very slow progression of AD.

ApoE was present within a majority of plaques immunoreactive for Aβ with the exception of very small Aβ deposits. In the senile plaques, cathepsin D is capable of proteolyzing both lipid-free and lipidated apoE to generate N-terminal and C-terminal fragments that are similar in size to those found in the AD brain (Zhou et al., 2006). As APP is extensively hydrolyzed by cathepsin D (Chevallier et al., 1997; Malik et al., 2011), this protease can be involved in the regulation of amyloid protein formation and play an important role in the pathogenesis of AD. Cathepsin D, being effective at neutral pH, can degrade cytoskeletal proteins such as neurofilaments, tubulin, and microtubule-associated proteins (Banay-Schwartz et al., 1983, 1987; Nixon and Marotta, 1984; Matus and Green, 1987; Johnson et al., 1991). Furthermore, cathepsin D-mediated degradation of apoE with the capacity of detoxifying hydroxynonenal (Zhou et al., 2006) will facilitate HNE induced carbonylation of Hsp70.1. In cultured Neuro-2a cells, Ji et al. (2002, 2006) demonstrated that apoE4, but not apoE3, potentiated lysosomal leakage and DNA fragmentation (Fig. 9g), and the lysosomal leakage was more induced by aggregated forms of $A\beta_{1-42}$ peptide than the soluble form (Fig. 9f). This is presumably because HNE being generated by Aβ-induced oxidative stress was not detoxified by apoE4, but by apoE3. Taken together, the increased enzymal activity and co-localization of cathepsin D in the apoE-positive senile plaques and neurofibrillary tangles (Zhou et al., 2006) may be closely related to neurodegenerative processes in AD (Bernstein et al., 1989; Cataldo et al., 1990; Cataldo and Nixon, 1990; Namba et al., 1991; Haas and Sparks, 1996; Metsaars et al., 2003).

6. Preventive and therapeutic strategies against Alzheimer's disease

There are likely to be behavioral (irregular physical and mental exercises, sedentary lifestyle), dietary (high-calorie, high-fat, high-linoleic acid), and other environmental (irradiation, electromagnetic waves, pollution) factors that may gradually affect the brain health. Although a causal relationship between these factors and AD occurrence has not been well established, the age-related cerebral arteriosclerosis and the resultant, mild but long-standing cerebral ischemia as well as concomitant oxidative stresses can undoubtedly affect survival of neurons (Fig. 10). Cerebral ischemia- and/or Aβ-induced calpain activation and ROS-induced HNE generation, when combined together, cause Hsp70.1 carbonylation and cleavage which ultimately lead to the lysosomal rupture. Based on the '*calpain–cathepsin hypothesis*', the author asserts such a possibility that inhibitions of calpain or cathepsin activity is likely to be an effective AD therapy. The administration of pharmacological agents that prevent calpain before the onset of AD course confers optimal neuroprotection. However, most patients are treated decades after the onset, when considerable numbers of hippocampal neurons have been already damaged by activated calpain and oxidants. Therefore, the pharmacological inhibition of post-calpain executioners of cell death; e.g. cathepsins B, L, or D might be more useful not to expand neuronal loss.

The efficacy of cathepsin blockade in AD animal models would greatly support that such therapy will be effective also for patients with AD. For example, cathepsin B has been proposed as an alternative candidate of β-secretase, because it produces Aβ by efficient cleavage of the wild-type β-secretase site of APP (Hook et al., 2005). Accordingly, deleting the cathepsin B gene in transgenic mice expressing human APP containing the wild-type β- and London mutant γ-secretase site (APPLon) sequences, caused a substantial reduction in brain Aβ, reduced brain plaque and improved memory deficits (Hook et al., 2009; Kindy et al., 2012). Moreover, administering the calpain–cathepsin inhibitor E64-d (l-3-trans-carboxyrane2; the membrane permeable analog of E64-c) (Murata et al., 1991; Towatari et al., 1991) to transgenic APPLon mice also improved memory deficits, by reducing brain Aβ and amyloid plaque, and providing potent neuroprotection (Hook et al., 2008, 2011). This group found that eliminating or inhibiting cathepsin B reduced brain Aβ in animals expressing APP containing the wild-type β-secretase site sequence but had no effect on animals expressing APP containing the Swedish mutant β-secretase site sequence (APPSwe). That lack of effect on Aβ in APPSwe mice was confirmed by Mueller-Steiner et al. (2006), but Trinchese et al. (2008) showed that nonetheless E64-d produced neuroprotective effects and improved memory deficits in APPSwe mice, being independent of Aβ lowering effect. Accordingly, irrelevant to Aβ, cathepsin B appears to be an effective drug target in most AD patients.

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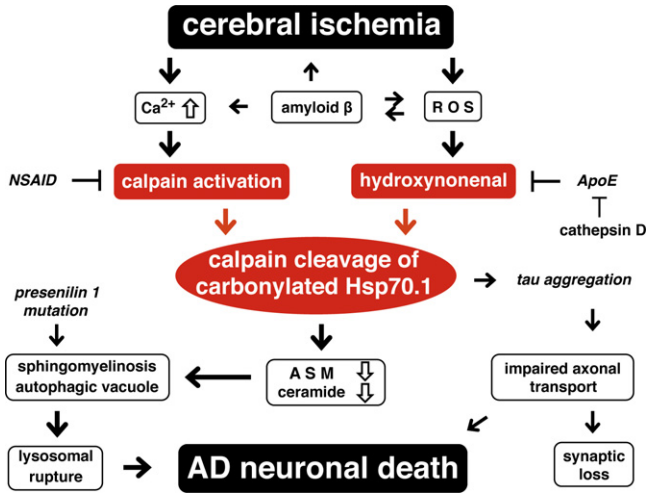

Fig. 10. Flow-chart of the molecular mechanisms involved in Alzheimer neuronal death. Neurons degenerating in AD exhibit perturbed cellular Ca^{2+} homeostasis, increased generation of ROS and depositions of Aβ due to the long-standing, mild cerebral ischemia with aging. Although numerous lines of genetic and biochemical evidence until now suggested that Aβ is central to the pathogenesis of AD, Aβ appears to be merely one of the causative factors. Instead, the 'calpain-mediated cleavage of carbonylated Hsp70.1′ appears to be crucial for the development of Alzheimer neuronal death. Hydroxynonenal (HNE) is a membrane lipid-derived neurotoxin that carbonylates Hsp 70.1 being indispensable for the lysosomal stabilization. As the Hsp70.1-mediated activation of acid sphingomyelinase (ASM) leads to an increment in ceramide for the membrane stabilization, it is probable in the Alzheimer brain that carbonylation and cleavage of Hsp70.1 may cause decrease of ASM activity and accumulation of sphingomyelin with the resultant lysosomal rupture. The calpain–cathepsin cascade would be exaggerated in the presence of Aβ but can proceed even without Aβ, and this can explain why AD occurs in those without any Aβ depositions in the brain. This cascade can reasonably explain implications of all players characterizing AD, including HNE, apoE4, Aβ, non-steroidal anti-inflammatory drugs (NSAID), presenilin 1, and tau for the Alzheimer neuronal cell death. For example, apoE4, the strongest genetic risk factor for the late onset sporadic AD, accelerates toxicity of HNE because of failure of binding or detoxification. Implications of other players were described in detail in the text.

The neuroprotective effects of cathepsin inhibition were first reported by the author's group in cerebral ischemia models (Yamashima et al., 1998; Tsuchiya et al., 1999), and have been discussed in various experimental paradigms (Ray et al., 2000; Wang et al., 2002; Tsubokawa et al., 2006; Anagli et al., 2008; Luo et al., 2010). The epoxysuccinyl peptides CA-074 (N-(L-3-trans-carboxyoxirane-2-cabonyl)-L-isoleucyl- L-proline) is known to be a specific inhibitor of cathepsin B, while E-64c (N-(L-3-trans- carboxyoxirane-2-carbonyl)-L-leucine-3-methylbutylamide) is known to inhibit cathepsins B and L and calpain (Hashida et al., 1980). Accordingly, the neuroprotective effect of E64-c is much stronger than CA-074; approximately 67% of hippocampal CA1 neurons were saved from delayed neuronal death by CA-074 on day 5 in monkeys undergoing 20 min brain ischemia (Yamashima et al., 1998), while approximately 84% of CA1 neurons were saved by E64-c in the same experimental paradigm (Tsuchiya et al., 1999). These studies on the non-AD models indicate that the neuroprotective effects of E64-c occur by the inhibitions of both calpain and cathepsins, rather than the Aβ lowering effect. Similarly, preictal administration of pyridoxal (Vitamin B_6) showed some neuroprotective effects against transient ischemia in both hippocampal and retina neurons, because the active aldehyde at position 4 of the pyridine ring has an affinity for the active site -SH of cysteine residues of cathepsins B and L (Yamashima et al., 2001; Wang et al., 2002). Accordingly, supplementary intake of Vitamin B_6 might, more or less, contributes to preventing Alzheimer neuronal death by inhibiting cathepsin B.

Since currently-available drugs such as acetylcholinesterase inhibitors and N-methyl-*D*-aspartate receptor antagonists are only for the partial symptomatic relief trying to slow deterioration of the patients, there is a significant need of new concepts for developing a novel strategy that can modify the underlying causative factors of the disease. The most promising preventive interventions in AD are preictal inhibitions of oxidative stresses, HNE production, and calpain overactivation, eventually for the blockade of 'calpain-mediated cleavage of carbonylated Hsp70.1′. Hsp70.1 has a pivotal role in regulating the life and death of neurons. Since 'calpain-mediated cleavage of cabonylated Hsp70.1′ is central in the pathogenesis of Alzheimer neuronal death, agents protecting Hsp70.1 from HNE carbonylation and calpain cleavage can serve as critical targets for the therapeutic drug development. However, given the known role of calpains in key cell processes such as mitosis, cell cycle regulation, differentiation, cell migration, adhesion, and signal transduction (Suzuki et al., 2004), nonspecific calpain inhibition is a potentially unacceptable therapeutic strategy. In this sence, NSAID, often utilized for the treatment of rheumatoid arthritis, may be useful for the calpain inhibition with little adverse effect (Raveendran et al., 2008; Silver et al., 2010) (Fig. 10).

Furthermore, for the protection of Hsp70.1 from the oxidative stresses, it seems prudent to develop drugs and other treatments that target HNE itself. Molecules that quench/detoxify HNE include glutathione-S-transferases, histidine analog (histidyl hydrazide), N-acetylcysteine, taurine, and carnosine derivatives as well as various anti-lipid peroxidation agents (Mattson, 2009). Energy-dense, high-fat 'fast-food' increases the plasma HNE significantly within minutes to hours, whereas dietary energy restriction reduces HNE production and enhances HNE detoxification (Johnson et al., 2007; Devaraj et al., 2008). Accordingly, changes of the dietary and lifestyle factors are essentially indispensable, and should be practiced consistently. One should avoid excessive intake of linoleic acids especially involved in soybean and corn oils, because these vegetable oils may generate abundant HNE when cooked at frying temperature (Seppanen and Csallany, 2006; Han and Csallany, 2008). Another promising therapeutic approach for AD is restoring functional Hsp70.1 proteins, for example, by the replacement therapy or transferring Hsp genes into the degenerating neurons. Of course, improvement of ASM function by gene therapy for preventing lysosomal lipidosis and the resultant rupture, if possible, becomes one of the therapeutic options.

The membrane lipid peroxidation product HNE is recently recognized as a particularly important mediator of cell degeneration and dysfunction in a range of disorders including cardiovascular disease, stroke, arthritis, diabetes, atherosclerosis, and asthma as well as AD (Mattson, 2009). However, the concept of HNE-induced lysosomal rupture was first described in this review as a cause of the Alzheimer neuronal death. Advances in the research of programmed neuronal necrosis using ischemic monkey experimental paradigms (Yamashima et al., 1996, 1998, 2003; Yamashima, 2000, 2004, 2012; Oikawa et al., 2009, Yamashima and Oikawa, 2009; Sahara and Yamashima, 2010; Zhu et al., 2012) promise rapid progress of therapeutic strategy for not only AD but also other neurodegenerative diseases in the very near future.

7. Concluding remarks

A continuum of abnormalities of the autophagic-lysosomal system can be identified in neurons of the AD brain. Lysosomes of the degenerating neurons prior to cell death often show accumulations of granulo-vacuolar degenerations on light microscopy while multilamellar phospholipidosis on electron microscopy. Although it is difficult to pinpoint the triggering event, recent data suggest that Alzheimer neuronal death may be triggered by long-standing, silent brain ischemia due to aging. The resultant activation of calpain over years as well as generation of HNE due to the oxidative stresses should be crucial for the development of

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autophagic-lysosomal failure. The extent of calpain activation and HNE generation as well as apoE's detoxifying capacity of HNE may determine individual risk for AD. Aβ will merely facilitate vulnerability of primarily ischemic neuronal cells by exaggerating calpain activation and HNE-mediated carbonylation of Hsp70.1. Calpain-mediated cleavage of the carbonylated Hsp70.1, via inactivation of BMP, leads initially to lysosomal sphingolipidosis, and ultimately to deprivation of ceramide at the lysosomal membrane. This causes destabilization and/or rupture of the lysosomal membranes with the concomitant release of cathepsin enzymes into the cytoplasm. Furthermore, Hsp70.1 dysfunction causes aggregation of phosphorylated tau and disturbance of the axonal transport. These would altogether impair the cytoprotection machinery being afforded by the elimination of potentially toxic oxidized proteins and membrane lipids. Accordingly, it is likely that the common molecular cascade exists between ischemic and Alzheimer neuronal death, and the latter occurs not by apoptosis but by programmed necrosis. A new insight into the Alzheimer neuronal death, based on the '*calpain–cathepsin hypothesis*' being formulated for ischemic neuronal death, would contribute not only to elucidating the molecular cascade of Alzheimer neuronal death (Fig. 10) but also to developing an effective AD therapeutic strategy.

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