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Review

Can 'calpain-cathepsin hypothesis' explain Alzheimer neuronal death?

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ABSTRACT

Neurons are highly specialized post-mitotic cells, so their homeostasis and survival depend on the tightly-regulated, continuous protein degradation, synthesis, and turnover. In neurons, autophagy is indispensable to facilitate recycling of long-lived, damaged proteins and organelles in a lysosome-dependent manner. Since lysosomal proteolysis under basal conditions performs an essential housekeeping function, inhibition of the proteolysis exacerbates level of neurodegeneration. The latter is characterized by an accumulation of abnormal proteins or organelles within autophagic vacuoles which reveal as 'granulo-vacuolar degenerations' on microscopy. Heat-shock protein70.1 (Hsp70.1), as a means of molecular chaperone and lysosomal stabilizer, is a potent survival protein that confers neuroprotection against diverse stimuli, but its depletion induces neurodegeneration via autophagy failure. In response to hydroxynonenal generated from linoleic or arachidonic acids by the reactive oxygen species, a specific oxidative injury 'carbonylation' occurs at the key site Arg469 of Hsp70.1. Oxidative stress-induced carbonylation of Hsp70.1, in coordination with the calpain-mediated cleavage, leads to lysosomal destabilization/rupture and release of cathepsins with the resultant neuronal death. Hsp70.1 carbonylation which occurs anywhere in the brain is indispensable for neuronal death, but extent of calpain activation should be more crucial for determining the cell death fate. Importantly, not only acute ischemia during stroke but also chronic ischemia due to ageing may cause calpain activation. Here, role of Hsp70.1-mediated lysosomal rupture is discussed by comparing ischemic and Alzheimer neuronal death. A common neuronal death cascade may exist between cerebral ischemia and Alzheimer's disease.

Contents

1. Background

Professor Christian de Duve, the Nobel Prizer in 1974, defined lysosomes as 'suicide bags' that can cause cell death and tissue autolysis upon rupture (de Duve, 1983). Although lysosomal rupture was recognized thereafter as a mechanism to kill cells (de Duve et al., 1974; Firestone et al., 1979), interests in implicating lysosomes for necrotic cell death faded during the following decades, and concomitantly lysosomal involvement in cell death was overlooked. These were largely because (1) there was no methodology to differentiate lysosomal rupture from the post-mortem alterations in autolytic cells, (2) lysosomal membrane destabilization does not necessarily change the ultrastructure of lysosomal membranes, and (3) the ability of methyl-ketone-based caspase inhibitors (e.g. zVAD-fmk) to inhibit cell death was considered as a proof for not 'necrotic' but 'apoptotic' cell death (Brunk and Ericsson, 1972; Aits and Jäättelä, 2013).

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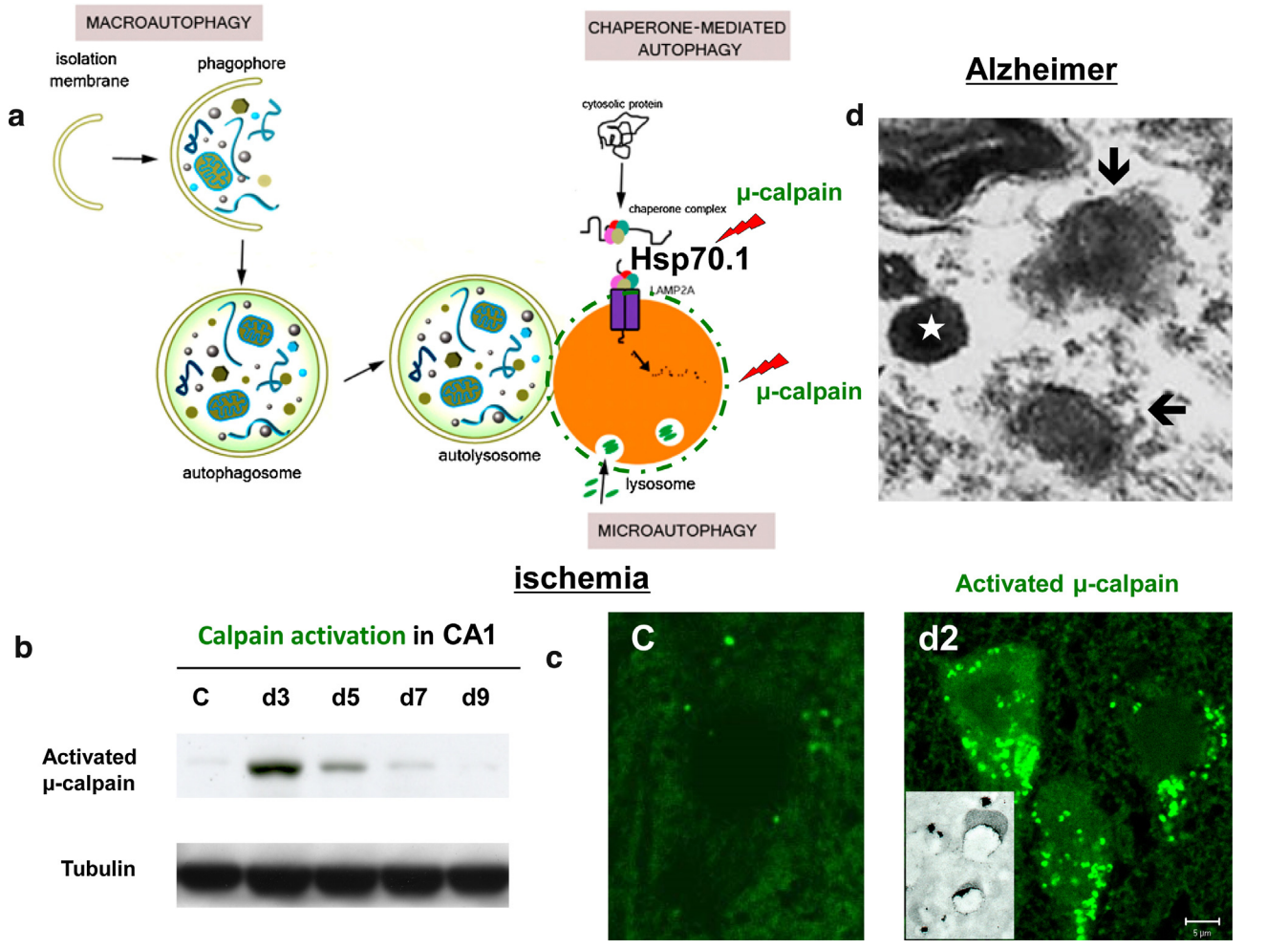

Fig. 1. (a) Schematic representation of the three types of autophagy: macroautophagy, chaperone-mediated autophagy, and microautophagy. Autophagy is a self-digestive process that ensures the lysosomal degradation of superfluous or damaged organelles and proteins for their turnover. Hsp70.1 functions for survival not only as a chaperone (cargo) protein but also as a stabilizer of the lysosomal membrane. (b) Western blotting of the hippocampal CA1 tissues shows maximal activation of μ-calpain on day 3 (d3) after transient ischemia. (c) The control CA1 neurons (C) show negligible immunoreactivity of activated μ-calpain. In contrast, on day 2 (d2) after transient ischemia, activated μ-calpain is immunostained as coarse granules (green) in the perinuclear cytoplasm. Immunoelectron microscopy shows that activated μ-calpain is localized at the lysosomal membranes (rectangle). The concept of 'calpain-mediated injury upon lysosomal membranes' emerged from this finding (Yamashima et al., 1996, 1998). Scale bar = 5 μm. (d) Double-membrane structures (arrows) indicating lysosomal membrane destabilization in the Alzheimer neuron. Please, note the difference from the configuration of the normal lysosome (asrerisk) (modified from Fig. 8b, Nixon et al., 2005).

The mechanisms underlying lysosomal membrane rupture or permeabilization (Fig. 1d) are largely ambiguous, possibly reflecting multiple means to destabilize the lysosomal membrane. However, interests in lysosomal cell death were revived recently, since the 'calpain-cathepsin hypothesis' corroborated the role of lysosomal rupture as an executor of programmed neuronal necrosis in the non-human primates (Yamashima et al., 1996, 1998, 2003; Yamashima, 2000, 2004, 2012, 2013; Yamashima and Oikawa, 2009; Zhu et al., 2014). Independent support for the interaction between calpain and cathepsin came from the unique work using *C. elegans* models published in *nature*, where loss of function in the proteases CLP-1 and TRA-3 (equivalent to calpains) as well as ASP-3 and ASP-4 (equivalent to cathepsins) was neuroprotective (Syntichaki et al., 2002). The 'calpain-cathepsin hypothesis' helps elucidate how impairments in proteolysis contribute to the development of neuronal dysfunction, degeneration and death from low-species animals to primates.

Calpain is Ca^{2+}-regulated cysteine protease, playing an important role in the regulation of cell death (Bevers and Neumar, 2008). As heat-shock protein70.1 (Hsp70.1), a major human Hsp70, simply Hsp70, also called Hsp72 or HSPA1, is involved in maintaining the lysosomal membrane integrity, reactive oxygen species (ROS)-induced Hsp70.1 carbonylation followed by calpain-mediated cleavage, combined together, cause lysosomal membrane rupture. Lysosomal hydrolytic enzyme, cathepsins, can degrade many cellular proteins and take over the role of 'death-executing proteases'. This review summarizes current knowledge on lysosomal neuronal death with an emphasis on Hsp70.1 that has dual effects as a chaperone of damaged/aged proteins and a stabilizer of lysosomal membranes.

2. Hsp70.1-mediated autophagy in neurons

2.1. Housekeeping function of autophagy

In the highly specialized post-mitotic cells such as neurons, the protein quality-control processes are directly linked to their homeostasis and survival. Removal of misfolded proteins, long-lived proteins, cellular macromolecules, protein aggregates, and damaged organelles, is crucial for maintaining the physiological function of neurons, because in the post-mitotic cells renewal of the cell constituents cannot be done through cell devision. Autophagy

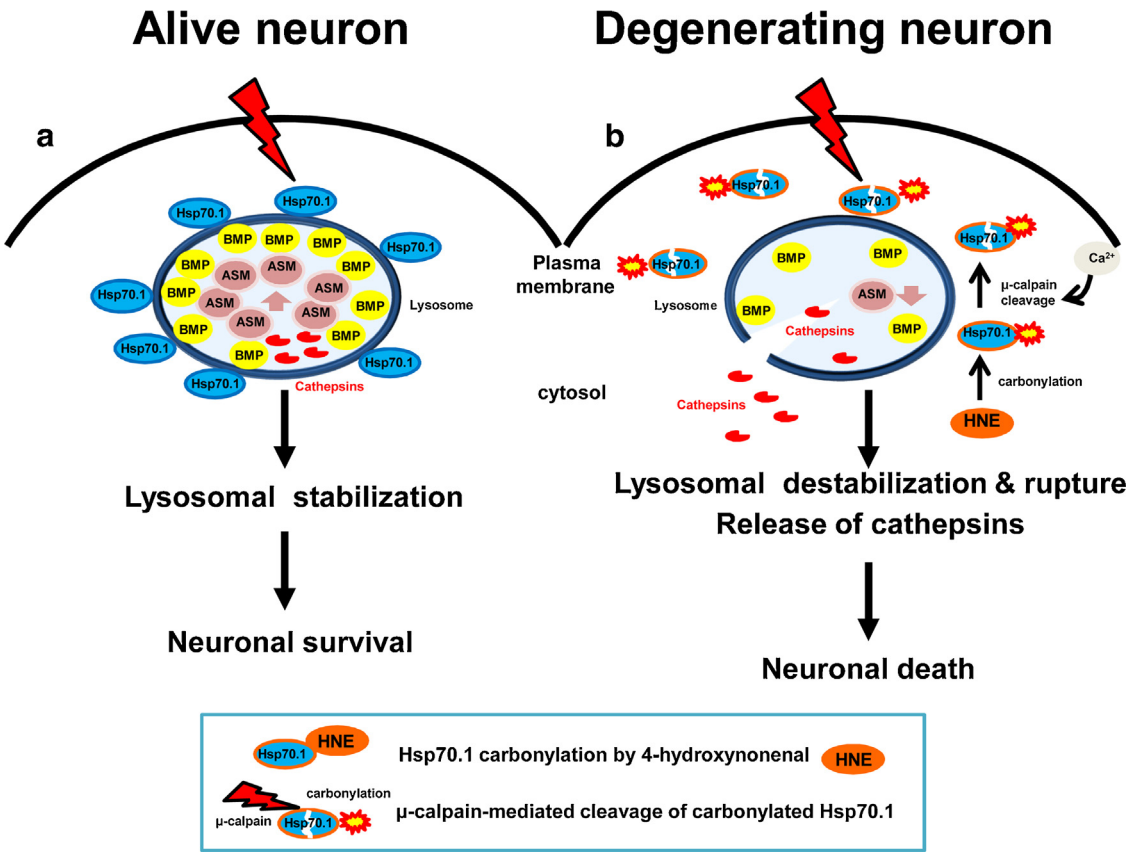

Fig. 2. Role of Hsp70.1 affecting neuronal survival (left) or death (right) by differentially regulating ASM (pink). (a) In the ischemia-resistant (alive) neurons, Hsp70.1 (blue), after working as a molecular chaperone, binds to BMP (yellow) to enhance ASM activity and facilitate the lysosomal stabilization. (b) In contrast, in the ischemia-vulnerable (degenerating) neurons, activated μ-calpain cleaves carbonylated Hsp70.1 to diminish Hsp70.1-BMP binding. This leads to a decrease in ASM activity, which induces lysosomal permeabilization/rupture with leakage of hydrolytic enzyme cathepsins. HNE (orange); hydroxynonenal (cited from Zhu et al., 2014).

(from Greek, meaning 'self-eating') is acting as an essential physiological system for maintaining cellular homeostasis (Fader and Colombo, 2009). In mammalian cells, different extracellular signals such as nutrient starvation or various stresses can trigger autophagy. Since lysosomes serve for the final degradation, their impairment increases the risk of autophagy failure.

There are three types of autophagy (Fig. 1a): (1) macroautophagy, (2) chaperone-mediated autophagy, and (3) microautophagy (Rubinsztein et al., 2005; Levine and Kroemer, 2008). (1) Macroautophagy is the process by which membrane engulfs cytosolic organelles and/or macromolecules to form an autophagosome, with the engulfed materials being delivered to the lysosome for degradation (Yoshimori, 2007). Importantly, localization of autophagosomes and lysosomes is different within the neuron; the former are distributed throughout the cytoplasm, while the latter are located mainly in the juxtanuclear cytoplasm. To form autolysosomes for degradation, autophagosomes being generated in dendritic, axonal, and synaptic regions, should be transported as far as to lysosomes in the juxtanuclear cell body. Accordingly, when dendrites or axons are filled with protein aggregates such as amyloid β and/or phosphorated tau proteins, autophagosomes that have formed in dendritic, axonal, and synaptic regions cannot move to fuse with lysosomes to degrade the engulfed materials (Nixon et al., 2005; Komatsu et al., 2007; Nixon, 2007). Then, cytosolic proteins/aggregates impair lysosomal digestion (Martinez-Vicente et al., 2008), and incomplete lysosomal digestion leads to a further increment of protein aggregates (Pickford et al., 2008; Yang et al., 2011; Wolfe et al., 2013; Coffey et al., 2014). (2) chaperone-mediated autophagy involves the chaperone Hsp70.1 and its co-chaperones that recognize misfolded, damaged, and aged proteins, then bind to the lysosome-associated membrane protein type 2A (Lamp-2A), and are translocated across the lysosomal membrane for the membrane stabilization (Periyasamy-Thandavan et al., 2009; Kirkegaard and Jäättelä, 2009, 2010; Nikoletopoulou et al., 2015 Kirkegaard and Jäättelä, 2009, 2010). Lastly, (3) microautophagy is engulfment of cytoplasmic cargo directly by the lysosomal membrane.

2.2. Roles of Hsp70.1 and its carbonylation

Intraluminal membranes of the functioning lysosomes are characterized by abundant lysosome-specific anionic phospholipid, bis(monoacylglycero) phosphate (BMP) (Fig. 2a) (Schulze et al., 2009; Kolter and Sandhoff, 2010). BMP is restricted to the internal membranes of late endosomes and lysosomes, and Hsp70.1-BMP binding is indispensable for stabilizing lysosomal membrane (Kirkegaard et al., 2010). Large amounts of BMP in the lysosomal internal membrane distinguish it from the limiting membrane. BMP is required for the sphingolipid degradation at the internal membrane in the acidic (pH4.5) compartment. BMP binds to acid sphingomyelinase (ASM) and stimulates its ability to hydrolyse sphingomyelin to generate ceramide (Linke et al., 2001a,b; Kolter and Sandhoff, 2005). Ceramide at the lysosomal membrane can stabilize lipid phases, i.e., microdomains, more efficiently than cholesterol (Massey, 2001; Xu et al., 2001). Accordingly, ceramide protects the lysosomal limiting membrane from rupturing (Kirkegaard et al., 2010; Petersen et al., 2010; Petersen and Kirkegaard, 2010), presumably because the increased concentration of lysosomal ceramide can facilitate fusion of lysosomes

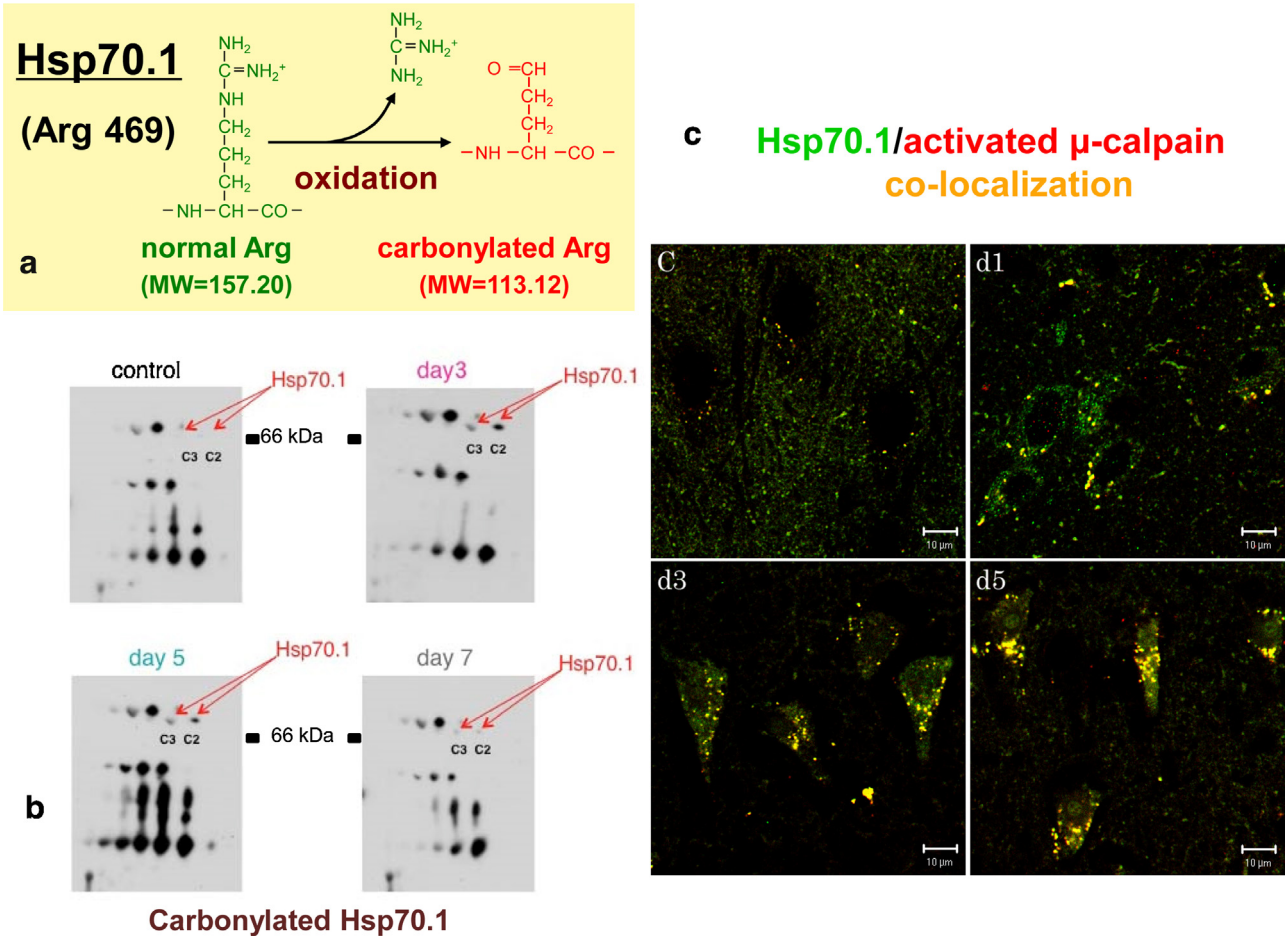

Fig. 3. HNE-mediated carbonylation of Hsp70.1 (a, b), and its colocalization with activated μ-calpain (c) in CA1 neurons. (a) In response to HNE being generated by the reactive oxygen species, carbonylation occurs at the key site, Arg469 of Hsp70.1. A decrease of its molecular weight from 157.20 to 113.12 indicates an insult by the oxidative stress (cited from Oikawa et al., 2009). (b) Two-dimensional carbonyl immunoblots of the postischemic CA1 tissues after immunoprecipitation with anti-Hsp70.1 antibody, shows upregulation of carbonylated Hsp70.1 on the postischemic days 3 and 5, compared to the control. (c) Hsp70.1 (green) and activated μ-calpain (red) (as well as Lamp-2, data not shown here), were shown to be colocalized (as shown in yellow) at the lysosomes by immunohistochemistry, especially on days 3 (d3) and 5 (d5) after ischemia (cited from Sahara and Yamashima, 2010). Scale bar = 10 μm.

with other intracellular vesicles and membranes, and strengthen limiting membranes (Heinrich et al., 2000).

The brain is highly sensitive to the ROS attack, because it has the highest metabolic rate and oxygen consumption among all organs of the body, a high content of unsaturated fatty acids, poor antioxidant defences, and high levels of iron (Chong et al., 2005). ROS have an unpaired electron which renders them highly reactive and causes oxidative damage to proteins, lipids, and nuclei acids. Accordingly, ROS being derived metabolically from molecular oxygen via the superoxide anion, attack proteins to form protein carbonyls, often with the resultant loss of function of the parent protein. Furthermore, ROS can attack polyunsaturated fatty acids (PUFA) in the membrane phospholipids, which results in the formation of lipid peroxides. Oxidative stress is strongly linked to perturbations of the autophagic pathway, and eventually to the development of neuronal dysfunction, degeneration and death that may underlie diverse neuropathological conditions. When blood flow to the brain is interrupted temporarily or permanently, for example, by acute stroke or chronic hypoxia, neurons undergo oxidative stress due to a series of molecular events such as excitotoxicity, mitochondrial dysfunction, acidotoxicity, ionic imbalance, and inflammation.

Hydroxynonenal (HNE) is generated from ω = 6 PUFA such as linoleic or arachidonic acids. In response to HNE being generated by the oxidative stress, a specific oxidative injury 'carbonylation' occurs at the key site Arg469 of Hsp70.1 in the postischemic monkey brain (Fig. 3a). A decrease of its molecular weight from 157.20 to 113.12 indicates oxidative injury of Hsp70.1 (Oikawa et al., 2009). Two-dimensional carbonyl immunoblots of the monkey hippocampal tissues after immunoprecipitation with anti-Hsp70.1 antibody, showed a remarkable upregulation of carbonylated Hsp70.1 in the CA1 sector after an ischemic insult (Fig. 3b). Furthermore, in the postischemic CA1 neurons, Hsp70.1 and activated μ-calpain were shown to be colocalized at the lysosomes by immunohistochemistry (Fig. 3c). In-vitro cleavage of Hsp70.1 occurred in response to activated μ-calpain in the various brain tissues of monkeys before and after HNE treatment (Fig. 4a). Intriguingly, Hsp70.1 cleavage occurred more drastically following HNE- or H_2O_2-mediated carbonylation not only in CA1, but also in other brain regions including hippocampal dentate gyrus (DG), cerebral cortex, cerebellar cortex and substantia nigra (Fig. 4a). Since the Hsp70.1 cleavage was blocked by a specific calpain inhibitor *N*-acetyl-Leu-Leu-Nle-CHO (ALLN) dose-dependently (Fig. 4b), it is likely that Hsp70.1 can be more efficiently cleaved by activated μ-calpain especially after carbonylation (Sahara and Yamashima, 2010; Yamashima, 2013; Yamashima et al., 2014). The CA1 neurons showing a remarkable Ca^{2+} mobilization compared to other brain regions, are more vulnerable to the ischemic insult (Yamashima et al., 1996). Accordingly, the cell death fate of neurons seems to be determined partly

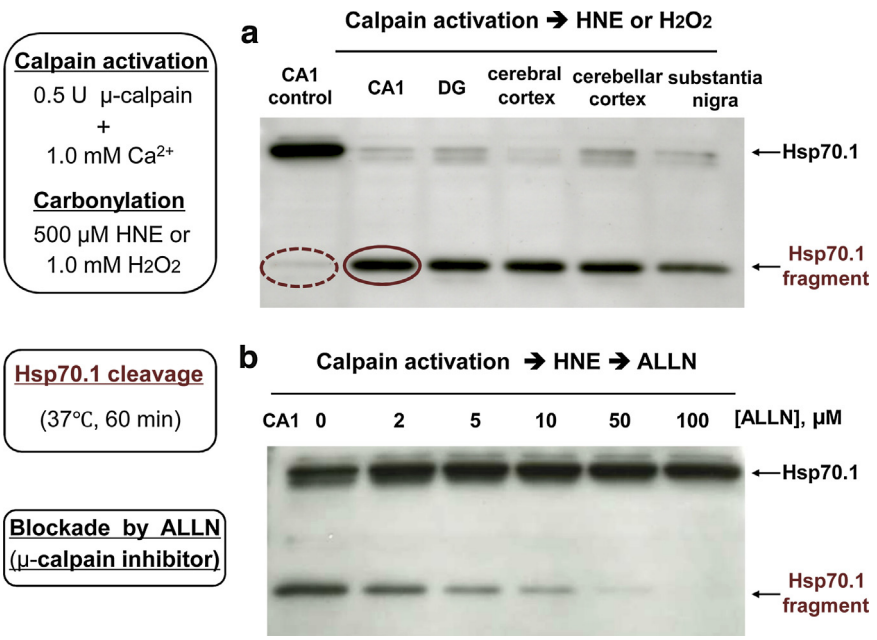

Fig. 4. *In-vitro* cleavage of Hsp70.1 by activated μ-calpain in various brain tissues from the non-ischemic monkey. (a) Without treatment with HNE or H_2O_2, 0.5U μ-calpain being activated by 1.0 mM of Ca^{2+} cleaved only a small amount of Hsp70.1 in the CA1 tissue (broken circle) and also in other brain regions studied (control bands of each not shown here). However, after carbonylation by 500 μM HNE or 1.0 mM H_2O_2, the cleavage of Hsp70.1 by calpain increased remarkably not only in CA1 (circle) but also in other brain regions. Both HNE and H_2O_2 showed facilitation effects upon calpain-mediated Hsp70.1 cleavage. Similar Hsp70.1 cleavage occurred also in the dentate gyrus (DG), cerebral cortex, cerebellar cortex and substantial nigra after treatment with 500 μM HNE or 1.0 mM H_2O_2. (b) Dose-dependent blockade upon calpain-mediated cleavage of carbonylated-Hsp70.1 was shown, using a specific calpain inhibitor N-acetyl-Leu-Leu-Nle-CHO (ALLN). Taken together, it is likely that calpain-mediated Hsp70.1 cleavage occurs especially after HNE-induced carbonylation. Extent of the calpain activation, not HNE carbonylation, appears to be a key factor determining the cell death fate (cited from Sahara and Yamashima, 2010: Yamashima et al., 2014).

by HNE but mainly by the extent of μ-calpain activation in each brain region.

Oxidative stress tends to be highly compartmentalized in the living subjects, and it is usually impossible to assess its levels in the human brain tissue. However, there is strong postmortem and experimental evidence concerning oxidative damage, especially by HNE, occurring in the Alzheimer's disease patients. For instance, elevated levels of HNE compared to age-matched controls, have been reported in the brain tissue (Markesbery and Lovell, 1998), ventricular fluid (Lovell et al., 1997), the amyloid component of senile plaques (Ando et al., 1998), and in the plasma of the Alzheimer patients (McGrath et al., 2001). Although the effects of HNE have been a focus of the recent research, the detailed mechanism of its effects upon neuronal death had been unclarified. Interestingly, recent evidence suggested that 'HNE-induced Hsp70.1 carbonylation' may be a new target for elucidating the mechanism of neuronal death (Oikawa et al., 2009; Yamashima and Oikawa, 2009), but whether and how HNE increases the risk of Alzheimer neuronal death are still not completely understood.

2.3. *Autophagy failure due to Hsp70.1 dysfunction*

Autophagy is involved in degradation of cellular components, including proteins and whole organelles within lysosomes; compartmentalized proteolytic sites containing acidic hydrolases. Cytosolic proteins and organelles are delivered to lysosomes for chaperone-mediated autophagy and macroautophagy. The complex Hsp70.1-cytosolic components binds to Lamp-2A (Cuervo and Dice, 1996), and after unfolding, substrate proteins are translocated into the lysosomal lumen for degradation, being assisted by a luminal form of Hsp70.1 (Cuervo, 2010) (Fig. 1a). Hsp70.1 is crucial not only as a molecular chaperone but also as a stabilizer of the limiting membrane (Kirkegaard et al., 2010). Accordingly, in case of Hsp70.1 dysfunction, failure of chaperone function and lysosomal autophagy as well as lysosomal permeabilization/rupture may occur (Figs. Fig. 1d, 2 b).

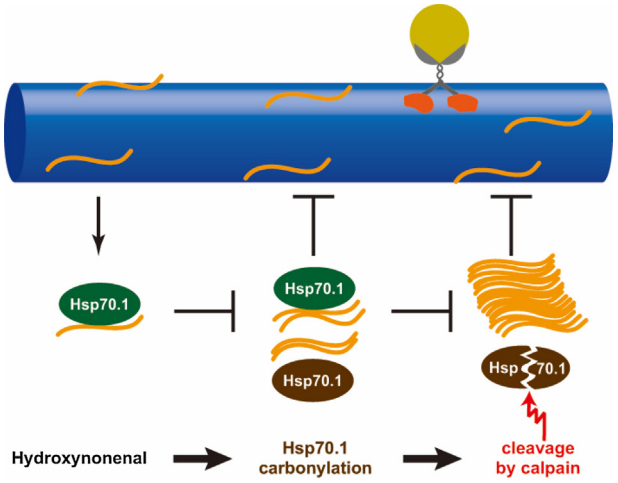

Fig. 5. Effects of carbonylation and cleavage of molecular chaperone Hsp70.1 on tau (orange curved bar) aggregation and axonal transport. Displacement of tau from the microtubule allows for its self-association and deposition. However, normal Hsp70 binds soluble, monomeric tau that dissociates from the microtubule, and prevents both tau aggregates and their inhibition on kinesin-dependent fast axonal transport. Accordingly, carbonylation and cleavage of Hsp70 lead initially to abnormal tau aggregation, and eventually to the impaired axonal transport (cited from Patterson et al., 2011b; Yamashima, 2013).

In endosomal microautophagy, extracellular material (nutrients and ligands) and plasma membrane receptors (including EGF receptors) are delivered to lysosomes for degradation via the endocytic pathway (Baixauli et al., 2014). Endosomal microautophagy occurs at multivesicular bodies (MVB), relying on the endosomal sorting complexes required for transport (ESCRT) I and III systems

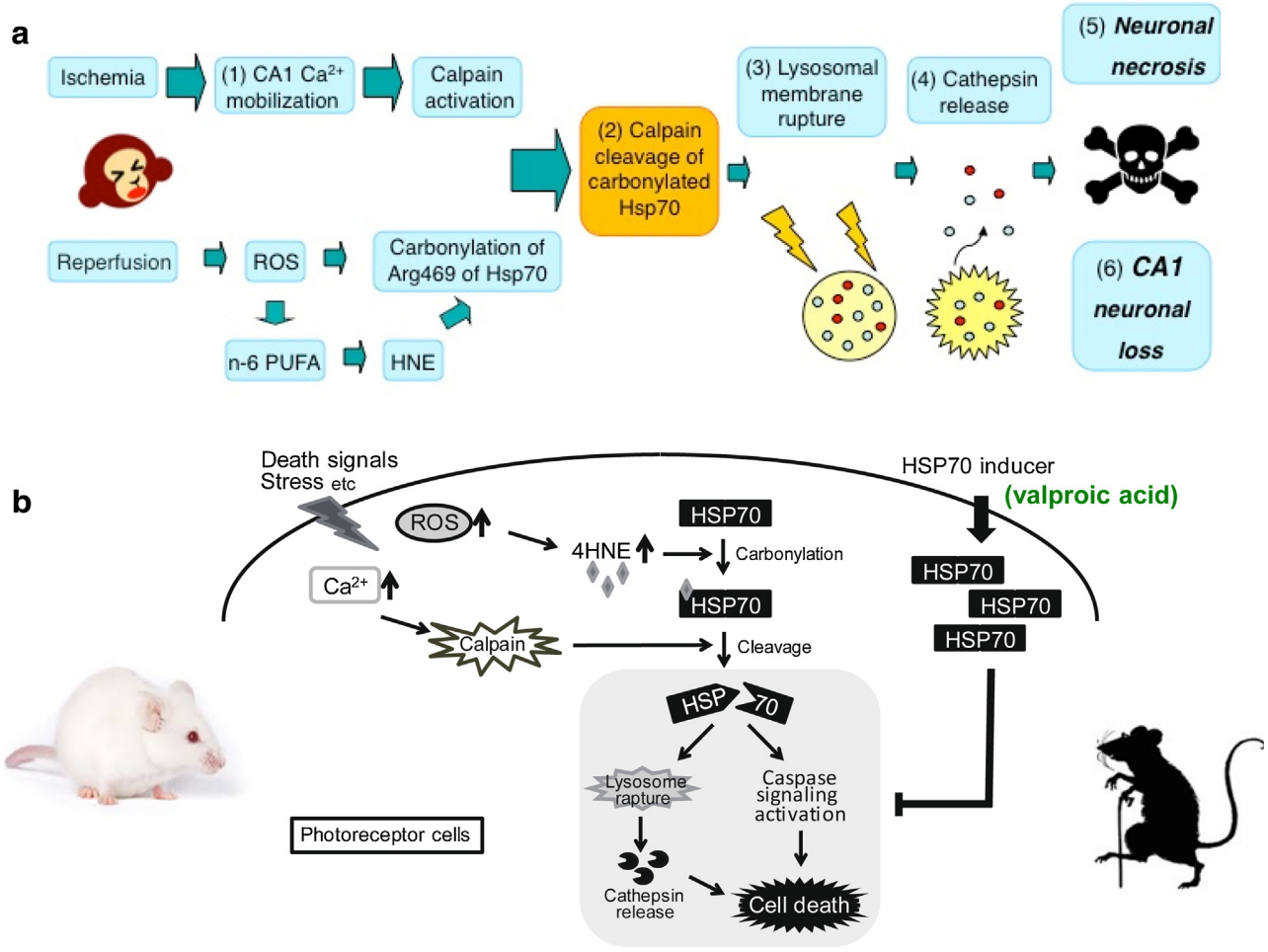

Fig. 6. A flow chart explaining the common calpain–cathepsin cascade in both (a) CA1 neuronal death after transient ischemia in monkeys (upper) and (b) photoreceptor cell death after the methylnitrosourea injection in mice (lower). During ischemia, Ca^{2+} mobilization (1) occurs specifically in the CA1 neuron, and μ-calpain is activated. During reperfusion, oxidation of n-6 (ω = 6) PUFA by the reactive oxygen species (ROS) produces HNE which carbonylates Hsp70.1 at the lysosomal membrane. Then, carbonylated Hsp70 (Hsp70.1 for humans) is efficiently cleaved by activated μ-calpain (2), and this leads to the lysosomal membrane destabilization/rupture (3). Consequently, release of hydrolytic enzyme cathepsins from lysosomes (4) occurs to induce programmed CA1 neuronal necrosis (5) within the CA1 sector (6) (cited from Yamashima and Oikawa, 2009). As shown in the lower column, the same cascade was demonstrated in the mice photoreceptor cell death (cited from Koriyama et al., 2014). Interestingly, Hsp70 inducer such as valproic acid; a histone deacetylase inhibitor, attenuated the mice photoreceptor cell death through inhibition of apoptotic caspase signals.

for the formation of vesicles. Cargo selection of soluble cytosolic proteins is mediated also by the chaperone Hsp70.1 at the vesicles of MVB (Sahu et al., 2011). Accordingly, in case of Hsp70.1 dysfunction, failure of endosomal microautophagy may occur as well (Fig. 1a).

The convergence between the autophagic and endocytic pathways are crucial in eukaryotic cells. Autophagy and the MVB pathway, combined together, are closely related with several intracellular events and are implicated in the cellular homeostasis. MVB fuse with autophagosomes to generate amphisomes which subsequently fuse with the functional lysosomes to generate autolysosomes. Amphisomes are more acidic (pH 5.7) than autophagosomes, probably due to the fusion of autophagosomes with endocytic vesicles carrying the proton pump. Finally, amphisomes acquire hydrolytic enzymes by fusion with lysosomes to form autolysosomes (Fader and Colombo, 2009).

Tau is a microtubule-associated protein predominantly expressed in axons, and plays an important role in the stabilization of microtubules. However, abnormally hyperphosphorylated tau sequesters normal tau and disrupts microtubules. In Alzheimer's disease, 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. However, Hsp70.1 prevents protein aggregation, and attenuates tau toxicity by maintaining tau in a soluble, non-aggregated state and by facilitating degradation of aggregated tau species (Dou et al., 2003; Petrucelli et al., 2004). Recently, Patterson et al. (2011b) demonstrated that Hsp70.1 directly inhibits tau aggregation by a preferential association with soluble, monomeric and prefibrillar oligomeric tau species, and prevents the toxic effect of preformed tau aggregates on anterograde fast axonal transport. Accordingly, Hsp70.1 disorders such as carbonylation and cleavage cause not only lysosomal stabilization but also tau aggregation, and contribute to neurofibrillary tangle formation (Fig. 5) (Patterson et al., 2011b; Yamashima, 2013).

3. Neurodegeneration and autophagic vacuoles

3.1. By what mechanism calpain causes neurodegeneration?

Calpain is a family of cysteine proteases that are ubiquitously expressed in most cell types, highly expressed in neurons, and involved in many brain functions. Elevated μ-calpain levels at anterior frontal lobes in the Alzheimer brain have been implicated in the amyloid precursor protein processing, neurofibril accumu-

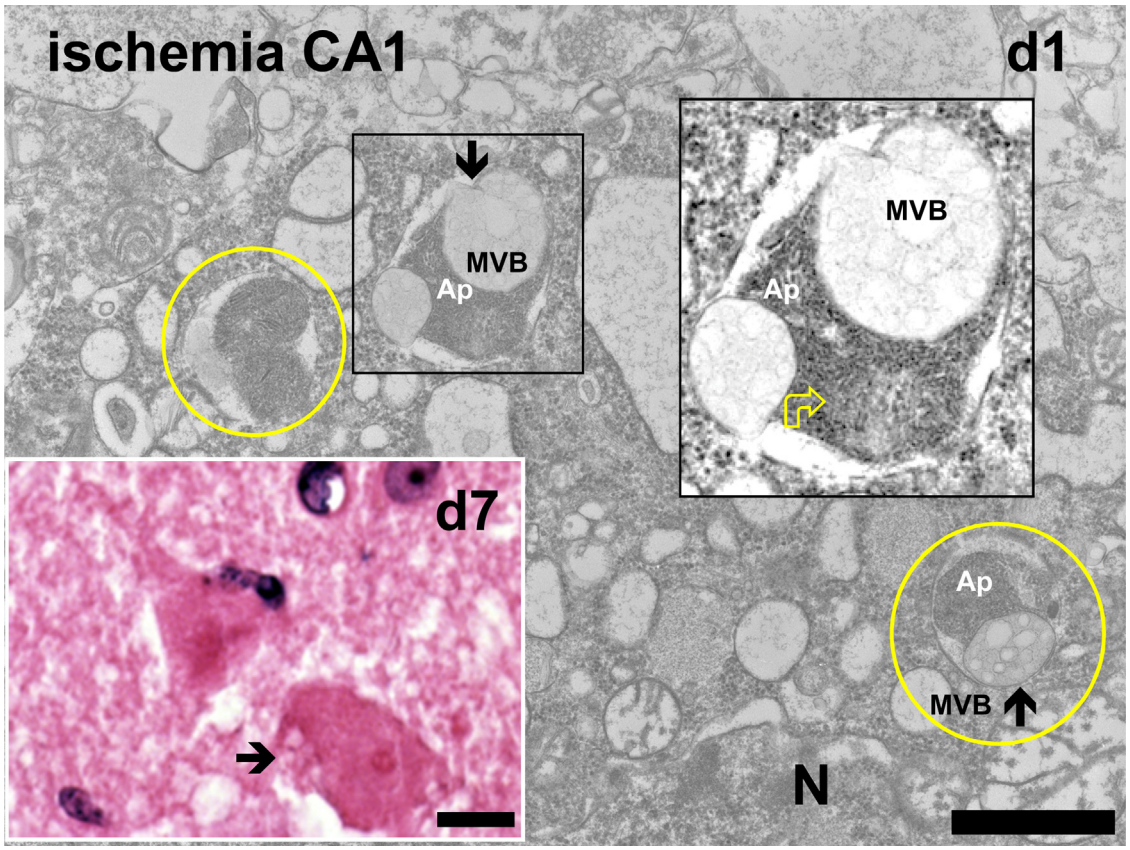

Fig. 7. By light microscopy (white rectangle), the postischemic day 7 (d7) CA1 neurons show eosinophilic coagulation necrosis with distinct microvacuolatory changes (arrow; hematoxylin-eosin staining, Scale bar = 10 μm). By electron microscopy, the postischemic day 1 (d1) CA1 neuron reveals early features of lysosomal vesiculosis as seen multivesicular body (MVB) (yellow circles). Please, note that an autophagosome (Ap) adjacent to MVB contains an engulfed mitochondria-like structure (curved arrow in the magnified black rectangle). N = nucleus, Scale bar = 1 μm.

lation, and neuronal death (Saito et al., 1993). Several forms of evidence have emerged since the early 1990s to support a role for calpain activation in the postischemic neurodegeneration (Saido et al., 1993; Roberts-Lewis et al., 1994; Neumar et al., 1996; Bartus et al., 1998). This was supported by a series of studies showing that neurodegeneration is prevented by μ-calpain inhibitors which were applied before (Lee et al., 1991; Rami and Krieglstein, 1993; Hong et al., 1994; Yokota et al., 1999) or after an ischemic insult (Bartus et al., 1994a,b; Li et al., 1998; Markgraf et al., 1998). Recent studies using μ-calpain-null mice showed decreases of Ca^{2+} influx and cortical neural degeneration after traumatic brain injury (Yamada et al., 2012). Calpain activation was closely related to neuronal death of non-human primates (Yamashima et al., 1996, 1998; Yamashima 2000, 2004).

To date, many calpain substrates have been identified in vitro, including caspases, Bax, Bid, AIF, etc., and some of them are known to play key roles in cell death (Wang, 2000). However, the precise mechanism linking individual calpain isoforms to oxidative injury and cell death pathways remained unclear. For the past 50 years after μ-calpain discovery (Guroff, 1964), much had remained unknown about the precise mechanism of calpain-mediated neuronal death and the specific calpain substrates in vivo. Despite multiple attempts at the substrate sequence analysis (Tompa et al., 2004; Cuerrier et al., 2005), no definitive method existed to predict whether a given compound is a calpain substrate in the brain. However, Sahara and Yamashima (2010) indicated from the in-vitro experiment that the specific calpain substrate in the non-human primate neurons should be a lysosomal membrane-associated protein, Hsp70.1. Furthermore, also in the mice photoreceptor cell death model, calpain-mediated cleavage of Hsp70 was confirmed to occur by HNE generation after the methylnitrosourea injection (Koriyama et al., 2014).

Although for the past decade chronic hypoxia has been focused to contribute to the development of Alzheimer's disease, role of hypoxia in its pathogenesis remains unelucidated. Recently, it was reported that chronic hypoxia treatment in the double transgenic Alzheimer model mice (APP/PS1 mice) accelerated not only amyloid pathology but also tau hyperphosphorylation. The latter was associated with a significant cyclin dependent kinase 5 (Cdk5)/p25 activation and upregulation of calpain (Gao et al., 2013). Cdk5 shows no enzymatic activity until it associates with its co-activator p35 which can be enzymatically cleaved to p25 by calpain. P25 contains all the elements necessary for Cdk5 binding and activation. It was shown in stroke rats that Cdk5 may be the principal protein kinase responsible for tau hyperphosphorylation (Wen et al., 2007). Taken together, activated calpain contributes to neurodegeneration conceivably by cleavage of not only Hsp70.1 (Fig. 5) but also p35 (Taniguchi et al., 2001; Wen et al., 2007; Gao et al., 2013).

3.2. *The 'calpain-cathepsin hypothesis'*

The lysosomal rupture-promoting effect of μ-calpain might be due to the calpain-mediated cleavage of Hsp70.1 which generates a protective ceramide shield on the lysosomal membrane by activating ASM. Accordingly, BMP down-regulation, combined with Hsp70.1 cleavage, presumably provoked impairment of ASM with accumulation of syphingomyelin and deficiency of ceramide at the lysosomal membrane (Yamashima, 2012; Zhu et al., 2014). These may lead to the lysosomal destabilization and/or rupture in both ischemic (Fig. 2b) and Alzheimer (Fig. 1d) neurons. Calpain activa-

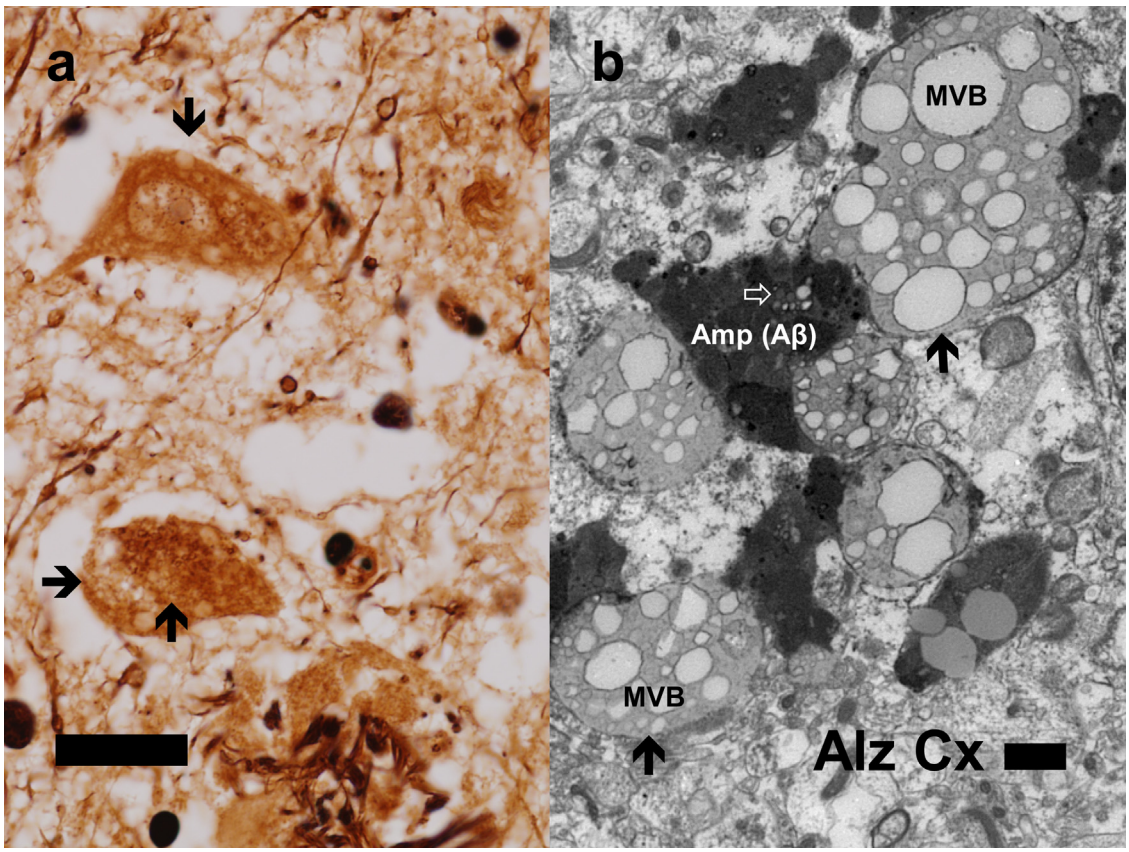

Fig. 8. In AD neurons associated with impaired lysosomal proteolysis, impairment of autophagy leads to accumulation of autophagic vacuoles (black arrows) with aggregates of microvesicles, being generally called 'granulo-vacuolar degenerations'. Scale bar = 20 μm. By the electron microscopic analysis, some autophagic vacuoles contain MVB within the cloudy substance (arrows). Conceivably, the neighbouring amphisome (Amp) containing amyloid β (Aβ) and also vesicles (open arrow) failed to fuse with MVB for the degradation (by the courtesy of Prof. Ralph A. Nixon, Nathan Kline Institute, New York). Scale bar = 1 μm.

tion (Fig. 1b) at the lysosomal membranes (Fig. 1c) may bring about programmed neuronal death through release of the hydrolytic cathepsin enzymes typically sequestered within lysosomes. This cascade, formulated by the author as the **'calpain–cathepsin hypothesis'** (Fig. 6a), consists of calpain-mediated disruption of lysosomal membranes followed by release of cathepsins into the cytoplasm. The 'calpain activation – Hsp70.1 depletion – cathepsin release' cascade may represent the central cascade for both ischemic and degenerative neuronal death (Yamashima and Oikawa, 2009; Yamashima, 2012, 2013; Yamashima et al., 2014). The most abundant lysosomal proteases released as a consequence of lysosomal rupture were cathepsins and some of them, including cathepsins B, L and D, remain active at the neutral pH (Katunuma and Kominami, 1983). Lysosomal cathepsins being released into the cytoplasm may damage cellular constitutive proteins and cytoskeletons. Simultaneously, they damage the lysosomal membrane itself 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).

Direct in-vivo evidence supporting a role of 'calpain-induced Hsp70.1 cleavage' in neuronal death is still insufficient. However, the observation of the spational colocalization (Fig. 3c) and molecular interactions between activated μ-calpain and the carbonylated Hsp70.1 (Fig. 4) may disclose the whole calpain–cathepsin cascade for neuronal death, and contribute to the development of the neuroprotection therapeutic strategy for various human brain diseases. Since Hsp70.1 carbonylation occurs in various brain regions (Fig. 4a) but neuronal death predominantly occurs in the vulnerable regions such as CA1, the extent of Ca^{2+} mobilization and calpain activation (Fig. 1b and c) is a key factor determining the cell death fate (Yamashima et al., 1996). Hsp70.1 has a pivotal role in regulating survival and death of neurons. However, Hsp70.1 carbonylation should be a supportive factor for determining the cell death fate, while calpain activation for the cleavage of Hsp70.1 is a principle factor. Importantly, not only acute ischemia during stroke but also chronic ischemia due to ageing may cause calpain activation in the Alzheimer brains (Taniguchi et al., 2001; Yamashima, 2013).

Since carbonylated proteins cannot be repaired once occurred, they are removed by calpain-mediated proteolytic degradation or accumulation as damaged or unfolded proteins in the cell (Stadtman and Berlett, 1998). The most promising preventive interventions in neuronal death are preictal inhibitions of oxidative stresses and HNE production eventually for the blockade of 'calpain-mediated cleavage of carbonylated Hsp70.1′. As both calpain and Hsp70.1 have physiological roles, protection and/or upregulation of Hsp70.1 rather than inhibition of calpain, might be reasonable for neuroprotection. Accordingly, agents protecting Hsp70.1 from HNE carbonylation and calpain cleavage served as critical targets for the therapeutic drug development. 'Calpain-mediated cleavage of cabonylated Hsp70.1′ is central in the pathogenesis of neuronal death from primates (Fig. 6a) to rodents (Fig. 6b) (Koriyama et al., 2014). It is intriguing that although transient, Hsp70 induction via transcriptional activation by a histone deacetylase inhibitor, valproic acid (Ren et al., 2004; Marinova et al., 2009), showed neuroprotective effects in the various experimental models (Pan et al., 2005; Lv et al., 2011; Xuan et al., 2012; Koriyama et al., 2014). Koriyama et al. (2014) have confirmed in mice that

Hsp70 is a crucial molecule in the methylnitrosourea-induced photoreceptor cell death.

3.3. Autophagic failure in ischemic and degenerating neurons

Understanding the intricate crosstalk between autophagy and cell homeostasis is essential, because this process can act as a pro-survival mechanism to inhibit neuronal demise. The role of autophagy during neuronal death is complex, because the autophagic process enables recycling of damaged cell constituents to promote cell survival but may result in cell death if overactivated (Hara et al., 2006). Since efficient autophagic degradation requires functional lysosomes with the aid of intact Hsp70.1, its disruption leads to autophagy failure. Declining autophagic-lysosomal degradative efficiency of cellular debris, causes accumulation of undigested lipids and proteins. This eventually leads to autophagic vacuole accumulation as a morphologic marker for neurodegeneration. A continuum of abnormalities of the lysosomal system can be identified in both the ischemic neurons (Fig. 6a) (Yamashima, 2013; Yamashima et al., 2014) and Alzheimer neurons (Fig. 1d) (Nixon et al., 2005). The common characteristic is that functional Hsp70.1 is indispensable for both MVB and lysosomal autophagy. Accordingly, in case of Hsp70.1 alteration, failure of both endosomal microautophay and lysosomal autophagy may occur, which leads to accumulation of autophagic vacuoles in both ischemic (Fig. 7) and Alzheimer (Fig. 8) neurons.

The Hsp70.1 +190 G/C polymorphism is located in the gene 5′UTR region and is implicated in alteration of the transcription binding factor. Boiocchi et al. (2015) have shown a statistically significant decrease of the C allele frequency of the Hsp70.1 +190 G/C polymorphism in Alzheimer patients. Hsp70.1 is highly stress-induced, while heat shock cognate (Hsc)71 is the constitutive isoform of Hsp70.1, being recruited by the cell as a primary defense against unfavorable conditions. By the proteomic identification of oxidatively modified proteins, Castegna et al. (2002) found an increased carbonylation of Hsc71 in the Alzheimer brains. By the redox proteomic analysis from the same group, elevated levels of the Hsp70.1 carbonylation were identified in the brains of mild cognitive impairment (MCI) which is the first stage of Alzheimer's disease (Sultana et al., 2010). As a cause of Hsp70.1 carbonylation, elevated levels of HNE was found in the plasma of Alzheimer patients (McGrath et al., 2001), while increased levels of HNE were found in the brain of MCI and early Alzheimer's disease (Williams et al., 2006). These findings, taken together, reinforce involvement of Hsp70.1 alteration in Alzheimer's disease. Inactivation of Hsp70.1 by the oxidative modification makes this protein unable to facilitate misfolded protein degradation, contributes to lysosomal destabilization, and eventually causes neuronal death associated with Alzheimer's disease (Yamashima, 2013; Yamashima et al., 2014).

Hsp70.1 is known to be an anti-apoptotic chaperone protein that is highly expressed also in the human cancers. Cancer cells upregulate Hsp70.1 to cope with their basal stressful conditions due to nutrient shortage, dysregulated growth, and accumulating unfolded proteins (Mjahed et al., 2012). Elevated Hsp70.1 expression in cancer cells of different origins seems to correlate with a faster disease progression (Rerole et al., 2011), whereas its inhibition has been shown to be an effective therapeutic strategy against cancer by inducing cell death (Nylandsted et al., 2002). During the disease processes, it is almost impossible to directly observe evidence of lysosomal rupture in the autolytic cancer cells. However, the morphologic feature of degeneration prior to cell death is a massive build-up of incompletely digested cell proteins and membrane lipids, being revealed as autophagic vacuoles. Hsp70.1-specific inhibitor, 2-phenylethynesulfonamide (Leu et al., 2009), induces caspase-independent cell death also in cancer cells by impairing lysosomal function. Granato et al. (2013) also reported that Hsp70.1 inhibition by 2-phenylethynesulfonamide induces lysosomal permeabilization with cathepsin D release and immunogenic cell death in primary effusion lymphoma (an aggressive B-cell lymphoma). Interestingly, lymphoma cells with Hsp70.1 inhibition showed similar empty vacuolatory changes as ischemic and degenerative neuronal death. These data altogether suggest that autophagic failure due to Hsp70.1 dysfunction is working for all type of necrotic cell death. It seems plausible to hypothesize that a common pathway might exist in all forms of cell degeneration.

4. Summary

This review summarized current knowledge on lysosomal neuronal death with an emphasis on a chaperone protein Hsp70.1. Stroke and Alzheimer's disease are the leading causes of adult neurological disability worldwide. The underlying cause for both is accepted to be neuronal death, but its molecular mechanism had remained unknown until the 'calpain-cathepsin hypothesis' was formulated in 1998. Although autophagy has important housekeeping or protective functions, extreme cell stimuli such as ischemia or diverse oxidative stresses trigger autophagy failure which disrupts cell function and ultimately leads to neurodegeneration.

Hsp70.1 stabilizes lysosomal membranes by binding to lysosomal lipid BMP, thus enhancing the activity of ASM, an important enzyme in the sphingolipid catabolism. Hsp70.1 essentially contributes to the cargo selection of cytosolic proteins at lysosomes, while Hsp70.1 also mediates that of membrane proteins at the vesicles of MVB. Accordingly, its impairment due to the oxidative injury causes dysfunction of autolysosomes and MVB, leading to autophagy failure. Autophagy induction to a mild oxidative stress may be neuroprotective, whereas excessive or chronic upregulation of autophagy conceivably promotes neuronal death via Hsp70.1 disruption and the resultant lysosomal rupture. Accordingly, the delicate balance is important between beneficial and deleterious upregulation in autophagy of neurons. In this sense, Hsp70.1 is a double-edged sword for determining neuronal survival or death.

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