Histol Histopathol (2002) 17: 897-908

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

Autophagy in neurons: a review

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Summary. Macroautophagy is a process of regulated turnover of cellular constituents that occurs during development and under conditions of stress such as starvation. Defects in autophagy have serious consequences, as they have been linked to neurodegenerative disease, cancer, and cardiomyopathy. This process, which exists in all eukaryotic cells, is tightly controlled, but in extreme cases results in the death of the cell. While major insights into the molecular and biochemical pathways involved have come from genetic studies in yeast, little is known about autophagic pathways in mammalian cells, particularly in neurons. Recently, research in neuronal culture models has begun to identify some characteristics of neuronal macroautophagy. The results suggest that macroautophagy in neurons may provide a neuroprotective mechanism. Here, we review the defining characteristics of autophagy with special attention to its role in neurodegenerative disorders, and recent efforts to delineate the pathway of autophagic protein degradation in neurons.

Key words: Autophagy, Vacuoles, Protein degradation, Apoptosis, Neurodegenerative disease

Introduction

The dynamic, tightly regulated balance between the formation and degradation of proteins and organelles maintains normal cell growth and development. The pathways by which most cytosolic proteins are degraded can be classified as either *lysosomal* or *nonlysosomal*. The nonlysosomal pathway is thought to be responsible for the highly selective degradation of cellular proteins under basal metabolic conditions. This pathway involves the targeting of susceptible proteins by ubiquitin, followed by hydrolysis via the proteasome. In contrast, lysosomal mechanisms are responsible for the degradation of extracellular proteins (endocytosis or pinocytosis) and the bulk degradation of intracellular proteins and organelles under conditions of stress (macroautophagy), although there is also lysosomal sequestration and degradation of some specific cytosolic proteins (microautophagy and chaperone-mediated autophagy).

The ubiquitin-mediated pathway is comprised of several essential components. The first is ubiquitin, a 76-amino acid polypeptide that is present in all eukaryotic cells. Ubiquitin is activated and then conjugated to target proteins via multi-step reactions catalyzed by E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin-protein ligase). Multiple ubiquitin molecules can be reversibly joined, via isopeptide bonds, to proteins that are destined for proteolysis and elimination. Eukaryotic proteins tagged with multiubiquitin chains are then selectively and irreversibly degraded by a large protease, the 26S proteasome complex, following the ubiquitin hydrolase-catalyzed depolymerization of polyubiquitin chains and release of free and reutilizable ubiquitin (Fig. 1). Substrates of the ubiquitin-proteasomal pathway are predominantly short-lived intracellular and plasma membrane proteins, as well as misfolded or damaged proteins in the cytosol, nucleus, or endoplasmic reticulum, and must be structurally distinct from neighboring stable and essential proteins. Some sequence elements (degradation signals) recognized by the ubiquitin system have been identified (for review, see (Ciechanover and Gonen, 1990; Hochstrasser, 1995, 2001; Yewdell, 2001)).

Autophagy, on the other hand, is likely the primary mechanism by which long-lived, stable proteins are degraded, and is the only mechanism by which entire organelles such as mitochondria and peroxisomes are recycled. Autophagy was originally described as a cellular response to starvation, and one of the primary functions of autophagy is to produce amino acids from degraded proteins for the survival of the cell when nutrients are scarce. Thus autophagy is stimulated by the decrease in amino acid content (Mortimore and Poso, 1986) and is hormonally controlled; glucagon promotes, while insulin inhibits, autophagy (Blommaart et al.,

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1997b). Autophagy is highly regulated in eukaryotic systems by a wide variety of factors including purines (Kovacs et al., 1981), growth factors (Ballard et al., 1980; Xue et al., 1999), serum (Amenta et al., 1978), adrenergic agonists (Seglen et al., 1990), and second messengers (Codogno et al., 1997). The complex regulation of autophagy affirms the importance of this pathway in mediating cell and tissue survival.

The autophagic/lysosomal pathway

The lysosomal pathway of intracellular protein turnover can be further divided into three distinct pathways in higher eukaryotes (Fig. 2): macroautophagy, chaperone-mediated autophagy, and microautophagy (Klionsky and Ohsumi, 1999).

Macroautophagy has been described as the main route for bulk protein degradation under conditions of nutrient starvation or stress. Genetic, biochemical and morphological studies have demonstrated that macroautophagy is a multi-step process. First, the autophagosome, a vesicular structure possessing a double membrane presumably derived from the endoplasmic reticulum, forms in the cytoplasm, engulfing various proteins, lipids, and damaged or dysfunctional organelles. The autophagosome later fuses with primary lysosomes (or vacuoles in yeast) which are comprised of a single membrane-bound compartment harboring a host of hydrolytic enzymes. The external membrane of the autophagosome becomes part of the lysosomal membrane upon fusion. Following fusion, the complex acidifies and matures into an autophagolysosome. The autophagosome and autophagolysosome are collectively referred to as autophagic vacuoles (AVs). Finally, the inner membrane structure within the autophagolysosome disintegrates while its contents are digested, and the vacuolar contents are recycled to provide amino acids and energy as needed by the cell (Fig. 2A; for review, see Dunn, 1990a,b, 1994; Stromhaug and Klionsky, 2001).

The proteins involved in macroautophagy have been well defined in yeast, and a subset of the genes involved are known as *apg* or *aut* genes (for review, see Klionsky and Emr, 2000; Thumm, 2000; Abeliovich and Klionsky, 2001; Ohsumi, 2001). The proteins involved in mammalian macroautophagy are less well known, although a number of mammalian homologues to the yeast apg proteins have been recently discovered. Examples include LC3 and beclin. LC3, the rat microtubule-associated protein 1 light chain 3, has a 28% amino acid identity with Apg8/Aut7p, which was previously determined to be essential for autophagy in yeast and thought to play a critical role in the formation of the autophagosome (Kirisako et al., 1999). Recently, the membrane-bound isoform of LC3 was specifically localized to autophagosome membranes and therefore has been suggested as a good marker for the detection of autophagosomes (Kabeya et al., 2000). Beclin 1, a Bcl-2-interacting protein, appears to be a mammalian homologue of the yeast autophagy gene *apg6* (Liang et al., 2001). The gene for beclin 1 is heterozygously deleted in 40-75% of sporadic human breast and ovarian

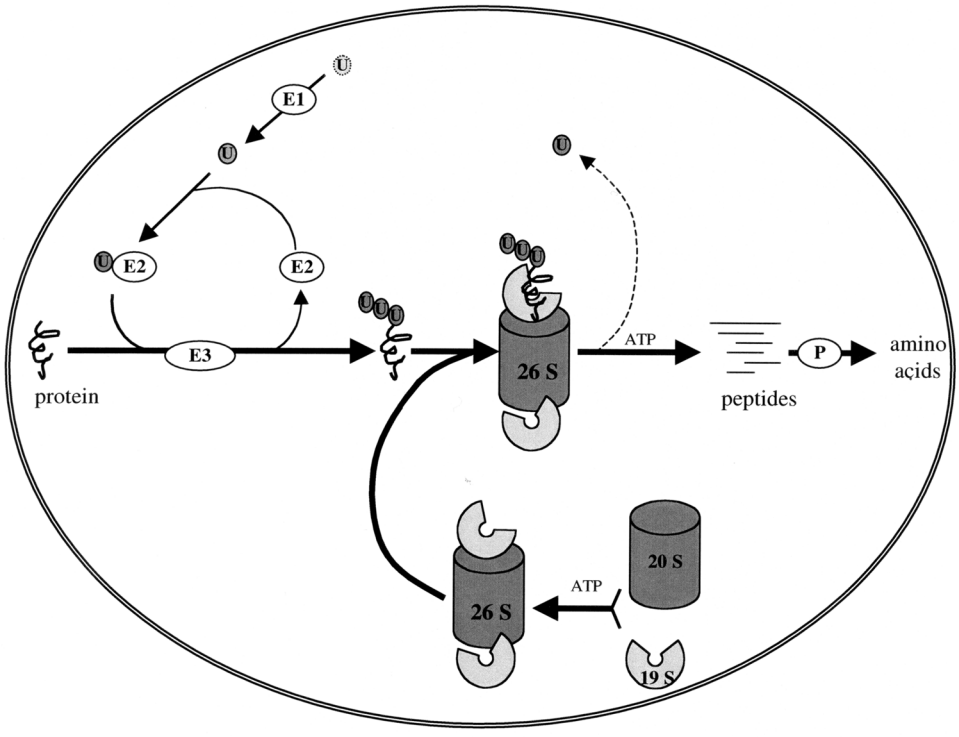

Fig. 1. The ubiquitin-proteasome pathway of protein degradation. Degradation of protein begins with the activation of ubiquitin (U). A ubiquitin molecule is activated by the ubiquitin-activating enzyme (E1), which then transfers the ubiquitin to the ubiquitin-carrier protein (E2). The E2-ubiquitin moiety is conjugated to the target protein by means of a ubiquitin-ligase enzyme (E3) such as parkin, resulting in the ubiquitination of the protein and the release of E2. Several ubiquitin molecules can be conjugated in this fashion to proteins targeted for degradation, and the selectivity for this process is believed to derive from the E3 enzyme. Following ubiquitination, the targeted protein is subjected to degradation via the 26S proteasome, formed from the coupling of the 20S and 19S subunits. The proteasome-mediated degradation process requires energy in the form of ATP, and results in the release of reusable ubiquitin, cleavage of the protein into peptides and amino acids through the actions of various peptidases and proteases (P).

cancers, and its protein product is generally lower in breast carcinoma epithelia than in normal breast epithelia. Beclin 1 was found to promote autophagy in human MCF7 breast carcinoma cells, resulting in inhibition of cell proliferation tumorigenesis in mice. In an earlier report, Beclin overexpression in murine neurons resulted in inhibition of Sindbi virus replication, protection against fatal Sindbi virus infection, and reduction of neuronal apoptosis (Liang et al., 1998). Although autophagy was not directly addressed in that report, a role for autophagy as a cellular defense mechanism was suggested in a follow-up study in which the herpes simplex virus-1 (HSV-1) neurovirulence protein, ICP34.5, directly antagonized the autophagic response normally elicited by PKR, a well-characterized antiviral molecule (Tallóczy et al., 2002). In other words, the autophagic degradation of dysfunctional, mutated, or viral (foreign) proteins is a normal host response to viral infection. However, HSV-1 has compensated for this defense mechanism via the ICP34.5-mediated inactivation of autophagy, allowing the virus to propagate. These findings introduce the idea that autophagy may play a protective role in eukaryotic systems.

While very little information has been garnered for proteins involved in neuronal macroautophagy, a role for the lysosomal enzyme cathepsin D has been suggested by the recent introduction of cathepsin D knockout mice. The CNS tissues of these mice are filled with autophagosomes and autophagolysosomes and contain ceroid lipofuscin (Koike et al., 2000), suggesting that cathepsin D is necessary for complete autophagic proteolysis. Moreover, upregulation of cathepsin D mRNA (Cataldo et al., 1995; Callahan et al., 1999) and enzyme activity (Schwagerl et al., 1995) has been associated with Alzheimer's disease (AD) brain, and cathepsin D has been shown to cleave tau, a protein associated with AD neurofibrillary tangles, in vitro (Kenessey et al., 1997). Since the upregulation of cathepsin D is an early and relatively specific observation in AD, some researchers believe that this enzyme has a role in the early stages of the neurodegenerative process (Saftig et al., 1996; Adamec et al., 2000), although whether its role is neuroprotective has not been examined.

Microautophagy, like macroautophagy, also involves the lysosomal incorporation of cytoplasmic proteins and inert particles, but is active under basal or well-nourished cellular conditions. In this pathway, proteins are internalized via direct uptake by the lysosomal membrane; this is accomplished by invagination of the membrane at multiple locations, forming a multivesicular body (Fig. 2B; Ahlberg et al., 1982; Dice, 1987). Microautophagy was originally believed to be

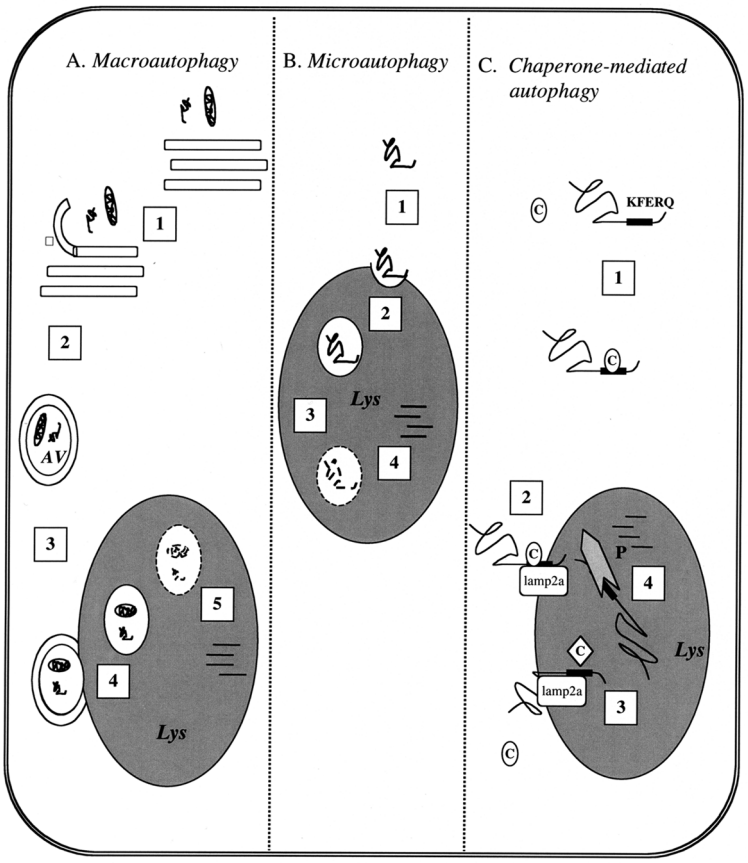

Fig. 2. The three pathways of autophagy. **A.** Macroautophagy. Proteins and organelles such as mitochondria are encircled by a membrane thought by some to originate from endoplasmic reticulum (1). These proteins and organelles are then sequestered, along with areas of cytosol, resulting in the formation of a double-membraned autophagic vesicle (AV) or autophagosome (2). The outer membrane of the autophagosome fuses with the lysosome (Lys; 3), releasing the inner vesicle into the lysosomal lumen (4), resulting in the formation of an autophagolysosome. Finally, the inner membrane structure disintegrates while the contents are digested by lysosomal enzymes (5). **B.** Microautophagy. Degradation of cytosolic proteins (1) occurs by the direct uptake of protein via invaginations of the lysosomal membrane (2), resulting in the internalization of protein and small soluble regions of the cytosol (3). The vacuolar membrane disintegrates and cytosolic components are degraded by lysosomal enzymes (4) as in macroautophagy. **C.** Chaperone-mediated autophagy. Cytosolic chaperone proteins (circled C) interact with target proteins containing a KFERQ sequence (1) and redirect these proteins to the surface membrane of lysosomes (2). Once there, the target proteins are bound to lamp2a and then transported, with the assistance of lysosomal chaperone proteins (diamond c), into the lysosome (3) for degradation by proteases (P) such as cathepsin (4).

non-selective, as the rates of internalization and degradation of labeled proteins and inert substances are comparable (Ahlberg et al., 1982; Marzella and Glaumann, 1987). Thus, microautophagy appeared to be responsible for the gradual, continuous turnover of cytosolic proteins that is not activated by nutritional deprivation or stress. However, evidence suggests that both macro- and microautophagy are also responsible for the preferential degradation of specific substrates (Lardeux and Mortimore, 1987; Masaki et al., 1987; Luiken et al., 1992; Yokota, 1993; Yuan et al., 1997).

An alternative pathway of lysosomal proteolysis, termed *chaperone-mediated macroautophagy*, was first identified in confluent, serum-deprived cultured fibroblasts and its defining feature is selectivity for distinct proteins (Dice et al., 1990). This process is restricted to the elimination of proteins that possess an amino acid sequence biochemically related to the pentapeptide Lys-Phe-Glu-Arg-Gln (KFERQ) during conditions of prolonged starvation (Chiang and Dice, 1988). Proteins containing this motif are recognized and bound by the cytosolic form of heat shock cognate protein of 73 kDa (hsc-73), a molecular chaperone (Terlecky and Dice, 1993; Cuervo et al., 1994). The selectivity of this pathway is further mediated by lgp-96 (also known as lamp-2a), a lysosomal integral membrane receptor of 96 kDa (Cuervo and Dice, 1996). This receptor binds to the target protein/chaperone complex and facilitates the transport of the targeted protein into the lysosomal matrix for degradation by proteases. Transport of the complex requires the assistance of a second chaperone protein, the lysosomal form of hsc-73 (Fig. 2C; Agarraberes et al., 1997; Cuervo et al., 1997).

The list of known substrates for chaperone-mediated autophagy include annexins, transcription factors, glycolytic enzymes, and cytosolic protease subunits (Cuervo et al., 2000). In fact, nearly 30% of cytosolic proteins contain the KFERQ-related motif (Dice, 1992), and it is likely that a diverse array of proteins are degraded by this pathway. Patients with Danon disease ("lysosomal glycogen storage disease with normal acid maltase") have mutations in the coding sequence of lamp-2, resulting in a deficiency in this receptor and an accumulation of autophagic material in a variety of tissues (Nishino et al., 2000; Saftig et al., 2001). Recently, lamp-2-deficient mice were developed and found to have increased mortality, extensive accumulation of autophagic vacuoles, and a remarkable impairment in the degradation of long-lived proteins (Tanaka et al., 2001).

Autophagy in cell death

In addition to its well-documented role in nutrient homeostasis, autophagy is also a prominent feature of cell death. Autophagy is responsible for the degradation of normal proteins involved in cellular remodeling found during metamorphosis, aging, and differentiation (Mortimore et al., 1996; Vittorini et al., 1999) as well as for the digestion and removal of abnormal proteins that would otherwise accumulate following toxin exposure, cancer, or disease. In some extreme instances of programmed cell death, cells can be completely degraded through autophagic digestion (Klionsky and Emr, 2000).

It has been suggested that autophagic (type 2) death is distinct from apoptotic (type 1) death (Clarke, 1990). The two pathways primarily differ with respect to morphology. Apoptosis is associated with nuclear and chromatin condensation, DNA fragmentation, organelle swelling, cytoplasmic vacuolization, and nuclear envelope disruption. Autophagy is correlated with the formation of autophagosomes, autolysosomes, electron-dense membranous autophagic vacuoles, myelin whorls, multivesicular bodies, as well as engulfment of entire organelles. However, a number of reports describe a considerable overlap between these two death modes in a variety of cellular systems (Xue et al., 1999; Bursch et al., 2000a,b; Terwel and Van de Berg, 2000; Uchiyama, 2001). For example, both apoptotic and autophagic cell death mechanisms occur during insect metamorphosis and are regulated by ecdysteroids (Li and Gilbert, 1999).

Differences in the gene regulation of these two forms of cell death have recently been reported in Drosophila (Lee and Baehrecke, 2001; Thummel, 2001). In the hawk moth, ecdysteroids directly trigger the autophagic, cell-autonomous death of specific motoneurons during metamorphosis, which involves caspase activation and the loss of mitochondrial function (Hoffman and Weeks, 2001; Weeks et al., 2001). In some systems, onset of mitochondrial permeability transition occurs prior to both apoptosis and autophagy (Lemasters et al., 1998).

Altogether, the above reports suggest that apoptosis and autophagy utilize some common regulatory mechanisms. Furthermore, the onset of autophagy can precede that of apoptosis and, in at least one report, delays apoptosis (Bauvy et al., 2001). Based in part on these results, it has been suggested that autophagy may play a protective role in the early stages of programmed cell death (Jellinger and Stadelmann, 2000a) as part of a desperate attempt by the cell to save itself from destruction. On the other hand, it is also plausible that the autophagic response is specific only to certain types of metabolic compromise (Nixon and Cataldo, 1995).

Autophagy in neurodegenerative diseases

One of the salient pathological features of chronic neurodegenerative disease is the slow, continual loss of cells within specific neuronal populations. Apoptotic cell death has been reported in AD, Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and various prion diseases, including Creutzfeldt-Jackob disease (for review, see (Offen et al., 2000) and (Martin, 2001)). However, Jellinger and Stadelmann have noted that the frequency of DNA fragmentation in PD and AD is too high to account for

the gradual neuronal loss associated with these diseases. In two recent reports, they hypothesize that autophagy may play a protective role in the early stages of programmed cell death (Jellinger and Stadelmann, 2000a,b).

A number of studies have demonstrated the presence

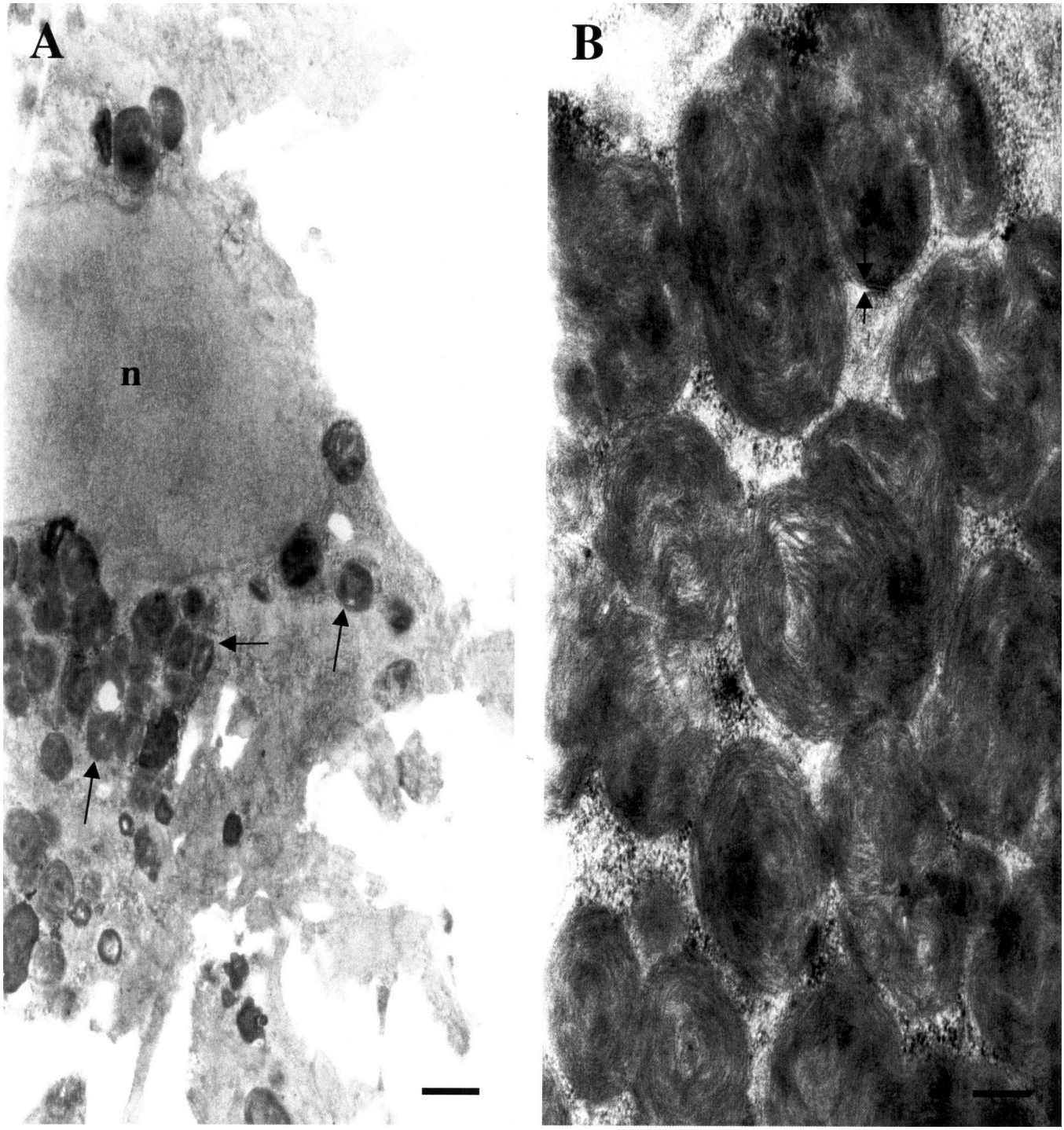

Fig. 3. METH induces the formation of autophagic membranous whorls in neurons. **A.** Electron micrograph of a ventral midbrain neuron exposed to 100 μM METH. Although the nucleus is healthy (n), the cytoplasm is filled with membranous whorls (arrows) while mitochondria and endoplasmic reticula are absent. Scale bar: 1 μm. **B.** A closer view of the membranous whorls. Some of these organelles are delimited by outer membranes (black arrows). Scale bar: 200 nm.

of autophagy in HD brain (Roizon et al., 1974, 1979) and in animal models of HD (Kegel et al., 2000; Petersén et al., 2001b). Likewise, autophagic degeneration was observed in AD (Cataldo et al., 1996; Stadelmann et al., 1999) and alterations in the lysosomal system were observed in both human disease and animal models of AD (Cataldo et al., 1994, 1995; Yang et al., 1998). Autophagic degeneration is prevalent in melanized neurons of PD brain (Anglade et al., 1997). Experimental scrapie and CJL, both prion-related diseases, promoted the activation of neuronal autophagy and formation of AVs (Boellaard et al., 1989, 1991). Finally, lipofuscin, the "aging pigment" that accumulates in epithelia and neurons of senescent organisms (Sohal and Brunk, 1989; Wolf, 1993) is associated with autophagocytosis (Terman and Brunk, 1998; Zhao et al., 1998). Interestingly, in HD brains, a very high accumulation of lipofuscin is found in comparison to normal brains (Tellez-Nagel et al., 1974).

Elucidation of neuronal autophagy in culture systems

Recently, a handful of reports have described autophagy in living neurons through the utilization of neuronal culture systems. The first such study we are aware of was on sympathetic ganglia treated with nerve growth factor (Hollenbeck, 1993). In this study, the cytosol of the cells was loaded with the fluorescent dextran TR-Dx, an inert marker that is sequestered by autophagy and delivered to lysosomes but not degraded (Hendil, 1981). Within 48 hours, the cytoplasmic dextran staining changed from an even, diffuse distribution to an exclusively punctate one consistent with lysosomal compartments. By electron microscopy, many of these organelles contained double membranes characteristic of AVs. When fluorescent dextran or other endocytic tracers were added to the culture medium, endocytosis was evident at the growth cone, and the organelles underwent net retrograde transport in acidic vesicles. Therefore, Hollenbeck concluded that there was an overlap of endocytic, autophagic, and lysosomal pathways in neurons.

The following year, we published analogous findings in cultured dopaminergic ventral midbrain neurons (Cubells et al., 1994). When these cultures were exposed to methamphetamine (METH), local oxyradical production was induced. This was followed by axonal swelling and formation of vacuoles in both axons and cell bodies. As in Hollenbeck's study, we found that the vacuoles were labeled by fluorescent dextran. Thus, we concluded that oxyradicals, perhaps due to oxidation products of dopamine (for review, see Sulzer and Zecca, 2000), elicited vacuole formation from endocytic organelles and lysosomes. The loss of neurites following METH appeared to result from the formation of the vacuoles. However, the direct evidence at the time of this study showed only that the vacuoles were involved in endocytosis. Further analyses of these cultured neurons have demonstrated that these organelles are

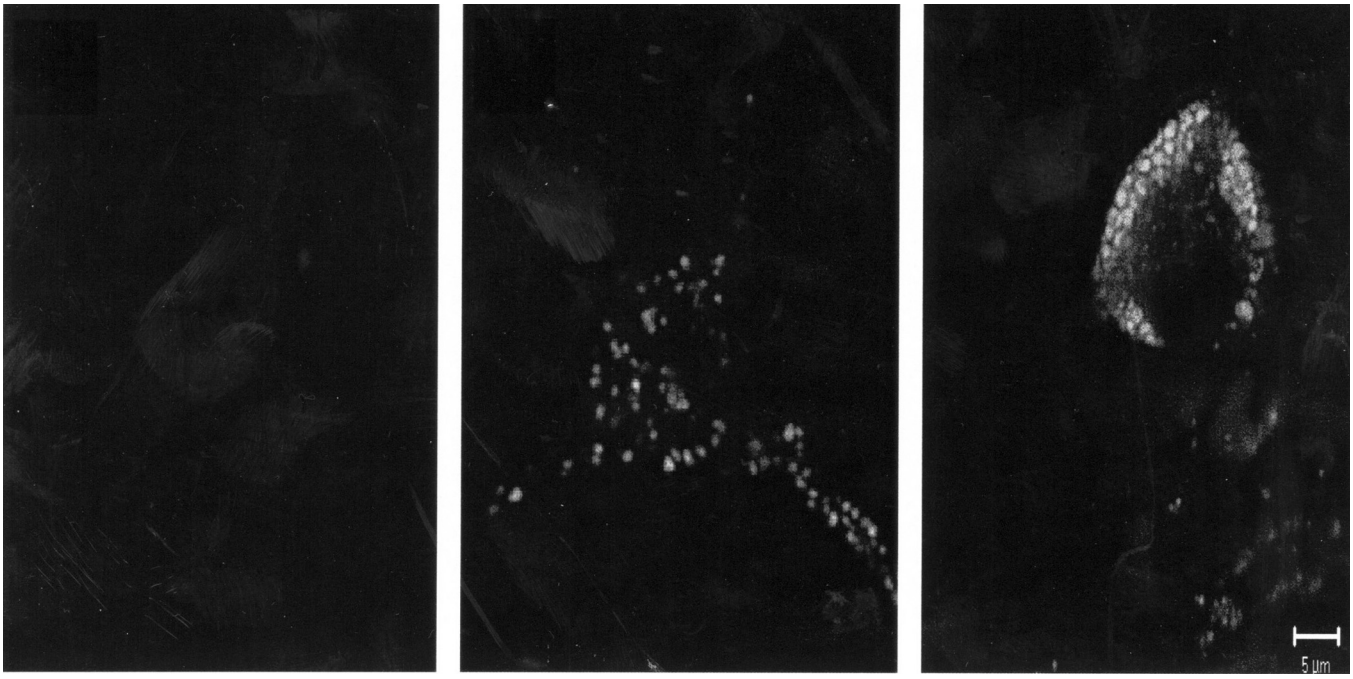

Fig. 4. METH-induced autophagic vacuole formation. Fluorescent micrographs of MDC-labeled autophagic vacuoles in ventral midbrain neurons. Neurons were treated with 100 μM METH for 0 **(A)**, 1 **(B)**, or 7 days **(C)**. MDC label accumulates in the cell body over time, but not in the nucleus. Scale bar: 5 μm.

AVs, as identified by the presence of double membranes, "membrane whorls", and by monodansylcadaverine labeling (see Figs 3, 4; Larsen et al., 2002). Therefore, both our work and Hollenbeck's suggest an overlap between endocytic and autophagic pathways in neurons, and that axonally-derived AVs may deliver their contents to the cell body for degradation. Moreover, these findings suggest that METH-induced neurodegeneration, which typically results in disappearance of neurites but not cell bodies, is due to autophagy.

In a separate study using these midbrain cultures, we discovered that dopamine oxyradical products were able to induce both autophagy and the formation of neuromelanin, the characteristic pigment that is a hallmark of PD (Sulzer et al., 2000). We found that the neuromelanin, a polymer primarily composed of dopamine or L-DOPA quinone products and their adducts, was located within AVs. Although the appearance of neuromelanin-engulfed organelles was very different from the membranous whorls elicited by METH, both were positively identified as AVs by electron microscopy. We also observed aspects of autophagy in another cell culture model of PD. Stable overexpression of mutant α-synuclein (A53T) in PC12 cells, a dopaminergic adrenal chromaffin pheochromocytoma-derived cell line, not only enhanced non-apoptotic cell death but also disrupted the ubiquitin-dependent proteolytic system and promoted the accumulation of AVs (Stefanis et al., 2001). Cell lines that expressed wild-type α-synuclein were devoid of these alterations, as were control cultures. Autophagy was also induced in PC12 cells following withdrawal of either serum (Ohsawa et al., 1998) or growth factor (Xue et al., 1999), two stimuli known to activate apoptotic cell death in this cell type.

Cell models of HD also reveal aspects of autophagic morphology. DiFiglia and colleagues showed that striata-derived immortalized neuronal cells, when transfected with full-length huntingtin with expanded CAG repeats (mutant) cDNA, developed vacuoles that were filled with cathepsin D and huntingtin protein, but not ubiquitin (Kegel et al., 2000). Ultrastructural analyses confirmed the presence of both early and late AVs, while wild-type cells and cells expressing truncated huntingtin were devoid of AVs. Biochemical analyses indicated that a substantial concentration of N-terminal fragments were associated with autolysosomes, suggesting that autophagy and/or lysosomal proteases may be involved in the proteolysis of huntingtin. We have also observed neuronal autophagy in primary striatal cultures comprised of GABAergic medium spiny neurons (Petersén et al., 2001b). In cultures derived from the R6/2 mouse line which expresses mutant huntingtin (Mangiarini et al., 1996), dopamine-induced oxyradical stress increased the levels of autophagy by 3-fold as compared to wild-type cultures. The AVs showed clear evidence of engulfment of a variety of cytosolic organelles and contained high levels of ubiquitin. Other studies have established that mutant huntingtin itself is ubiquitinated, both in HD striatum (DiFiglia et al., 1997; Mende-Mueller et al., 2001) and in transfected cell lines (Bence et al., 2001) and suggest that aggregation of mutant huntingtin may impair the ubiquitin-proteasome system (Jana et al., 2001). Altogether, these data suggest that both the proteasome and AVs are involved in the proteolysis of mutant huntingtin.

In another HD study, this time utilizing R6/1 mice, it was found that the striatal neurons derived from these animals developed partial resistance to dopamine-induced neurotoxicity with age (Petersén et al., 2001a). However, there were no differences in the levels of the established antioxidant defense systems, such as ascorbate, glutathione, and uric acid. At the ultrastructural level, dopamine administration reduced the density of synapses in both wild-type and R6/1 striata, but R6/1 mice displayed a greater number of dense lysosomal-like bodies within the dopamine-treated neurons, whereas dopamine exposure induced a more severe depletion of cytoplasmic organelles in wild-type neurons. Further EM analyses revealed the presence of autophagy in R6/1 striata at the time of resistance to toxins (Å Petersén, personal communication). As autophagy has been demonstrated, in some cases, as an attempt by cells to protect themselves from death (Clarke, 1990), the authors propose that mutant huntingtin may itself initiate autophagy in order to save striatal neurons from low-grade, long-term toxicity elicited by (endogenous) oxidized dopamine (Petersén et al., 2001a).

The above studies are consistent with the hypothesis that neuronal autophagy is elicited by stress, and in some cases, apoptotic stimuli. Moreover, the evidence to date is consistent with a neuroprotective role of macroautophagy. For example, the formation of neuromelanin in AVs sequesters oxyradicals in an impermeant state away from the cytosol. It is also plausible that upregulation of autophagy in neurodegenerative disease is a consequence of defects in the lysosomal and proteasomal degradation system, although this hypothesis has yet to be examined.

Detection of neuronal autophagy

Detection of autophagy can be achieved by ultrastructural microscopy, fluorescent markers, biochemical techniques, and pharmacological agents:

1. The primary and universally accepted method for detection of autophagy is through morphological characterization, using electron microscopy techniques. The development and/or presence of electron-dense AVs (with double membranes), membranous whorls, residual bodies, multivesicular bodies, as well as engulfed organelles can only be seen at the ultrastructural level. For example, we have been able to detect autophagy by electron microscopy in a variety of cellular models of PD (Sulzer et al., 2000; Stefanis et al., 2001), HD (Petersén et al., 2001b) and following METH

administration to primary dopaminergic neurons (Fig. 3, Larsen et al, 2002).

2. The development of lysosomal- and other vacuolar-specific fluorescent compounds has aided considerably in the detection of autophagic processes. One class of these compounds is fluorescent lipophilic weak bases that become membrane-impermeant in their protonated form, thereby accumulating in acidic organelles. Examples of such dyes are LysoTracker Red and acridine orange. Alternatively, fluorescent endocytic tracers such as LysoSensor Yellow/Blue Dextran are endocytosed by cells and transported in lysosomes. Another marker which has received much attention recently is monodansylcadaverine (MDC). This autofluorescent compound can be used to exclusively identify AVs in vivo (Biederbick et al., 1995; Petersén et al., 2001b) and in vitro (Fig. 4, Larsen et al., 2002; Petersén et al., 2001b). The mechanism by which MDC accumulates in AVs is believed to be a combination of ion trapping in acidic compartments and interaction with AV double membrane lipids (Niemann et al., 2000). While MDC has proven useful in that it demonstrates AVs in both living and fixed neurons (Petersén et al., 2001b), some have observed that it occasionally overlaps with conventional lysosomal markers such as the LysoTracker dyes (Katunuma, 1989).

3. An established biochemical method for quantitating autophagy is by measurement of long-lived protein degradation at serial time points following an appropriate stimuli. Generally, cells are labeled with a radioactive amino acid such as [^{3}H]leucine or [^{14}C]valine prior to the induction of autophagy (e.g. starvation). The degradation of long-lived proteins is then measured at various time points, following the degradation of short-lived proteins and removal of unincorporated label, by calculating TCA-precipitable radioactivity using an established formula (Gronostajski and Pardee, 1984). Enhanced proteolysis of long-lived proteins is an indicator of increased autophagy (Liang et al., 1999). A less selective means for identifying AVs is via biochemical markers coupled with, in some cases, centrifugation techniques. For example, subcellular fractionation on sucrose density gradients combined with antibody-mediated identification of lysosomal marker enzymes such as acid phosphatase and cathepsin (Biederbick et al., 1995) have been used to characterize MDC-labeled cellular compartments. Likewise, lactate dehydrogenase (LDH) is often used as an autophagosome marker in conjunction with centrifugation, for autophagosomal LDH is sedimentable and easily separated from cytosolic LDH during purification of organelles (Stromhaug et al., 1998). Although not conclusive by themselves, these procedure are useful when comparing the localization of various cellular markers in conjunction with morphological observation, and has aided in understanding the formation and development of AVs. Other biochemical characteristics are also useful in the detection of autophagy. Autophagy requires energy in the form of ATP (Kovacs and Kovacs, 1980), is temperature sensitive (Gordon et al., 1987), and requires intact cytoskeletal elements such as microtubules and microfilaments (Kovacs et al., 1981; Russell et al., 1989; Aplin et al., 1992; Dash and Moore, 1993).

4. Pharmacological characterization of macroautophagy has been achieved mostly through the inhibition of phosphatidylinositol 3-kinases (PI3K). In mammalian cells, administration of the class III PI3K inhibitors, wortmannin and LY294002, was found to halt macroautophagic degradation of proteins at the sequestration step (Blommaart et al., 1997a; Petiot et al., 2000). Similarly, 3-methyladenine, an agent long known to selectively and potently inhibit autophagy-dependent protein degradation and suppress the formation of autophagosomes (Kovacs et al., 1981; Seglen and Gordon, 1982; Blommaart et al., 1997a), was recently classified as a class III PI3K inhibitor (Petiot et al., 2000). Conversely, inhibition of class I_A PI3K stimulates autophagic sequestration, while activation of class I_A PI3K, through cytokine IL-13 treatment, resulted in the inhibition of macroautophagy (Petiot et al., 2000). The selectivity of 3-MA has been called into question, however, as it has been shown to inhibit autophagy and delay apoptosis following two independent death stimuli in sympathetic neurons (Xue et al., 1999), suggesting a convergent pathway in cell death. Another xenobiotic which has received much attention lately in the autophagy field is rapamycin, an inhibitor of the PI3K homologue, TOR (target of rapamycin). Rapamycin has been shown to activate autophagy in yeast (Blommaart et al., 1995; Noda and Ohsumi, 1998) and in mammalian cells (Cutler et al., 1999), even in the presence of nutrient-rich medium (for review, see (Sakai and Ogawa, 1982; Plomp et al., 1987; Mizushima et al., 1998)). Although TOR has been shown in yeast to repress starvation-specific transcription by promoting the sequestration of several nutrient-responsive transcription factors in the cytoplasm (for review, see Shmelzle and Hall, 2000), the exact mechanisms by which TOR and other PI3K homologues regulate autophagy have not yet been delineated.

Conclusions

Activation of autophagy occurs under physiological conditions (e.g. maintenance of equilibrium between protein/organelle biosynthesis and degradation/turnover), during development (e.g. pruning or removal of unnecessary tissue undergoing embryogenesis), and following pathological insult (e.g. prolonged or extreme exposure to toxic insult or disease situations). While the primary function of autophagy is to convert cellular macromolecules into energy substrates and metabolic building material at the expense of cell size, other functions may be provided by autophagy, including

inhibition of malignancy and protection of the cell from oxyradicals and mutated proteins. In neurons and neurodegenerative disorders, recent evidence suggests that macroautophagy could play a neuroprotective role.

Since inappropriate or prolonged activation of autophagy can lead to the complete demise of the cell, it is imperative that the regulation of autophagy is tightly controlled. Much of what is currently known about the process of autophagy has been garnered from studies in nutrient-deprived yeast and liver cells. Autophagy is highly conserved in a variety of eukaryotic organisms and the biochemical pathways involved are now being delineated. However, there are many questions left unanswered, particularly those concerning the underlying mechanisms and roles involved in neuronal systems.

Acknowledgements. The authors would like to thank Mary Schoenebeck for excellent electron micrographs, Drs. Anna Marie Cuervo, Beth Levine, and Janis Weeks for critique of the manuscript, and our colleagues, Drs. Patrik Brundin, Åsa Petersén, Eugene Mosharov, and Dmitriy Markov, for discussion and insight. The authors' own work cited here was funded by the National Parkinson's Foundation, the Parkinson's Disease Foundation, NINDS, NIDA, the Hereditary Disease Foundation, and the Huntington's Disease Society of America.

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Accepted March 13, 2002