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Review

Autophagy and Its Possible Roles in Nervous System Diseases, Damage and Repair

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ABSTRACT

Increased numbers of autophagosomes/autophagic vacuoles are seen in a variety of physiological and pathological states in the nervous system. In many cases, it is unclear if this phenomenon is the result of increased autophagic activity or decreased autophagosome-lysosome fusion. The functional significance of autophagy and its relationship to cell death in the nervous system is also poorly understood. In this review, we have considered these issues in the context of acute neuronal injury and a range of chronic neurodegenerative conditions, including the Lurcher mouse, Alzheimer's, Parkinson's, Huntington's and prion diseases. While many issues remain unresolved, these conditions raise the possibility that autophagy can have either deleterious or protective effects depending on the specific situation and stage in the pathological process.

INTRODUCTION

The ubiquitin-proteasome and autophagy-lysosome pathways are the two major routes for protein and organelle clearance in eukaryotic cells. Proteasomes predominantly degrade short-lived nuclear and cytosolic proteins. Poly-ubiquitination generally serves as a signal for proteasomal substrate recognition.[1] Since substrates need to be unfolded to pass through the narrow pore of the proteasome barrel, this precludes clearance of large membrane proteins and protein complexes (including oligomers and aggregates). Mammalian lysosomes, on the other hand can degrade substrates like protein complexes and organelles.[2] The bulk degradation of cytoplasmic proteins or organelles is largely mediated by macroautophagy, generally referred to as autophagy. It involves the formation of double membrane structures called autophagosomes/autophagic vacuoles (AVs), which fuse with lysosomes to form autolysosomes (also called autophagolysosomes) where their contents are then degraded by acidic lysosomal hydrolases.[3] Autophagosomes are generated by elongation of small membrane structures known as autophagosome precursors. However the precise origin(s) of these structures has yet to be elucidated (Fig. 1).[4] Autophagy can be induced under physiological stress conditions such as starvation. Several protein kinases regulate autophagy, the best characterized being the mammalian target of rapamycin (mTOR), which negatively regulates the pathway.[5] While the focus of this review is on macroautophagy, lysosomes can also directly engulf cytoplasm by invagination, protrusion and/or septation of the lysosomal limiting membrane, a process called microautophagy. Another pathway for cytosolic protein clearance via lysosomes is chaperone-mediated autophagy. This pathway is selective for specific cytosolic proteins that contain a pentapeptide motif. This motif is recognized by the chaperone heat shock cognate 70 (Hsc70), which transfers protein substrates to the lysosomal membrane, where, through binding to the receptor lysosome-associated membrane protein 2a (lamp2a), they are translocated into the lysosomal lumen and degraded.[6]

Increased numbers of AVs are a feature of a number of neurological diseases, certain neuronal cell death pathways and after neuronal injury. In many cases it is unclear if this is due to increased autophagic activity (i.e., induction of autophagosome formation) or decreased autophagosome-lysosome fusion (resulting in decreased removal of autophagosomes) (Fig. 2). The role of autophagy when it occurs coincidently with cell death is also unclear in many cases. If the increased autophagosomes are due to increased autophagic activity, is this directly contributing to the cell death, protecting against cell death, or occurring as an epiphenomenon driven by a pathway perturbed or contributing to the cell death (Fig. 3)?

Here we aim to put some of these questions into perspective in reviewing the current understanding of autophagy and its roles in the nervous system.

AUTOPHAGY AND INJURY

The phenomenon of autophagy in injured neurons was first described many years ago, being especially noted following axotomy or excitotoxicity. Autophagy was also observed after drug-induced block in nerve conduction or axonal transport, to some extent models of axotomy due to paralysis of axonal function (reviewed in ref. 7). Axotomy may cause neuronal cell death, especially in embryonic or neonatal animals, as this is the period when neurons are dependent on their targets for the supply of survival factors. However, in mature animals, where survival factors are derived locally or via autocrine loops, axotomy of peripheral neurons and motoneurons can lead to a robust regenerative response without any neuronal death.[8-14] In both cases, autophagy has been reported to be markedly enhanced, thus leading to two opposing theories: (1) that autophagy is a mechanism that clears the way for neuronal regeneration or (2) that it is a medium for cell destruction. The notion that autophagy is beneficial to survival is consistent with the evolutionary role of autophagy as a cell-autonomous nutritive mechanism,[15] while the role of autophagy in cell death is consistent with the ability of autophagy to dismantle cellular organelles, especially after stress or injury (a kind of protection as well).[16-18] There is no reason why both roles cannot be performed depending on cell context. Even caspases, normally causal in apoptosis, may be important for cell survival under certain conditions where death by autophagy may prevail.[19,20]

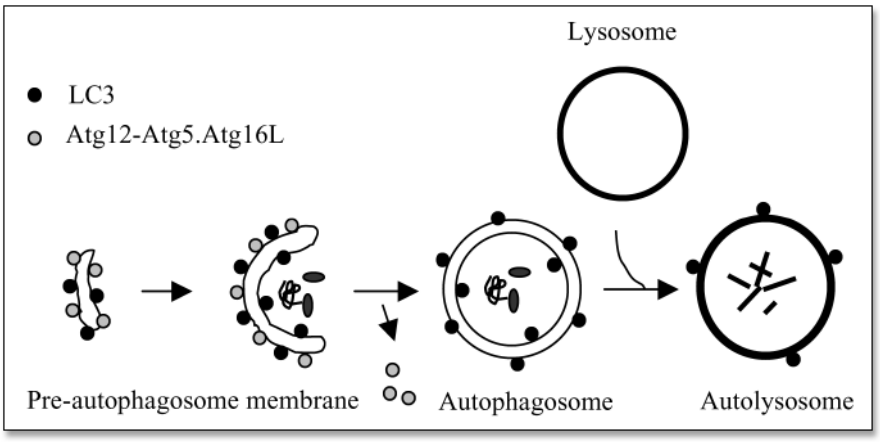

Figure 1. Macroautophagy in mammalian cells. A portion of the cytosol is enclosed by elongation of small membrane structures to form the autophagosomes/autophagic vacuoles (AVs). The outer membranes of AVs then fuse with lysosomes to form autolysosomes where their contents are degraded. Atg12-Atg5.Atg16L localize to the pre-autophagosome membranes, however dissociate upon completion of autophagosome formation. LC3-II (Atg8 homolog) is recruited onto autophagosome membrane throughout the process.

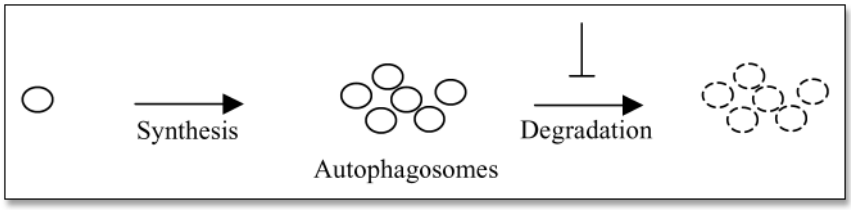

Figure 2. Autophagosome number is a function of synthesis and autophagosome-lysosome fusion/degradation. Increased numbers of autophagosomes can result from induction of autophagy but also from failure of autophagosome fusion with lysosomes.

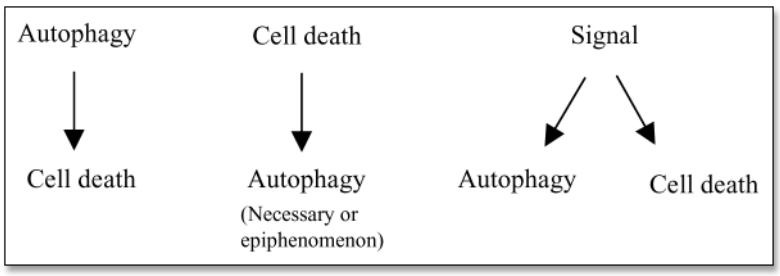

Figure 3. Possible theoretical relationships between autophagy and cell death, when they occur coincidently.

AUTOPHAGY IN LIVING NEURONS

The increase in autophagic activity after axotomy in living neurons is concomitant with chromatolysis, a stereotypic response where an area of the cytoplasm becomes largely devoid of organelles, especially ribosomes, and fills with numerous vesicles of various types, some of which have been described as autophagosomes.[21] The autophagic response occurs rapidly. For example, axotomy of superior cervical ganglion (SCG) neurons in adult rats results in increased autophagic activity in the cell body within two hours.[12] In this case, the response peaks within two days, concomitant with the very beginnings of regenerative responses. The contents of the vesicles in the cell bodies include mitochondria and Golgi/ER, classical substrates of autophagy, but many contain dense core vesicles associated with neurotransmitter synthesis. Thus, it was suggested that in the cell body, the autophagic activity clears away those aspects of neuronal function that are not concerned with the production of material for axon re-growth. A surge in vesicle formation, including autophagosomes, was also observed in the proximal stub of the axon that remains connected to the cell body, from whence regeneration will arise.[13] As mitochondria were found inside many autophagosomes, it was proposed that these need to be cleared before remodeling of the axon could begin. Remarkably, the number of vesicles of various morphologies remained significantly elevated in the neurons too up to 143 days, long after regeneration was nominally complete. The role of this extended reaction, and whether it finally abates, is not clear. In chromatolysis, it was noted that the response was lessened the more distant the insult occurred from the cell. In addition, nerve block was less efficacious at inducing this response compared to nerve cut.[22] Hence, the signals that induce autophagy may be linked to the severity of damage, though what these signals are is not known. Because excitoxicity can under some circumstances also provoke an

increase in autophagy, as detailed below and elsewhere,[23] damage due to pathological effects of calcium may be a common factor. However, though calcium has been implicated in some cases of autophagy[24,25] no direct causal relationships between calcium-induced signals and injury-induced autophagy have been described, though a calcium-regulated involvement in sequestration of synuclein by autophagic vesicles has been recently inferred[26] (see below).

AUTOPHAGY AND CELL DEATH

Newly axotomized neurons from the SCG of neonatal rats are dependent on nerve growth factor (NGF) for their survival. Despite a strong regenerative response, little autophagy was observed in the cell bodies during the first week of culture in the presence of NGF. However, culture without NGF induced a 20–30-fold increase in vesicular content, some of which are autophagosomes,[27,28] concomitantly with the induction of apoptosis. The vesicle reaction was prolonged if the neurons were not allowed to die by apoptosis (using a pan-caspase inhibitor). This reaction was not due to withdrawal of trophic support (i.e., starvation) since it occurred to similar extents in cell bodies of neurons induced to undergo apoptosis in the presence of NGF (through use of cytosine arabinoside that activates a dominant p53-dependent death pathway).[29] Given that loss of mitochondria was observed after a few days of incubation in the absence of NGF,[30] and these mitochondria no longer maintained a membrane potential, it was hypothesized that autophagy is responsible for disappearance of functionally-failed mitochondria, as proposed previously.[31] This idea was strengthened by the observation that bafilomycin A1, a V-type H^+ ATPase inhibitor that alkalinizes the lysosomal lumen, prevented the loss of mitochondria. However, the mechanisms and origins of these vesicles is still unclear, as rapamycin, the inhibitor of mTOR and an inducer of autophagy in many systems,[32-34] causes no changes in autophagic response in these neurons in the presence of NGF (though it blocks S6 kinase activity and S6 phosphorylation, Goemans C, unpublished observations) while the commonly used autophagic inhibitor 3-methyladenine, that blocked mitochondrial removal, was found to inhibit not only PI-3 Kinase activity important for autophagosome formation,[35] but also the cell death signalling pathway mediated via JNK and p38, and mitochondrial dysfunction.[27] Moreover, the mammalian Atg8 homologue MAP-LC3 II, a molecular marker of autophagosomes,[36] failed to segregate with any of the vesicles. Thus, whether autophagy plays a causal role in cell death in this model of axotomy is not clear. It may be that GABARAP (gamma 2-subunit of GABA-A receptor associated protein) and GATE-16 (Golgi-associated ATPase Enhancer of 16 kDa), the two other Atg8 homologues, play a role in autophagosome formation in neurons, as at least GABARAP has been described to associate within intracellular membranes such as the Golgi apparatus and postsynaptic cisternae,[37] though evidence for its association with autophagic vesicles is still lacking. Interestingly, GABARAP and GATE-16 were recently described as binding partners for ULK1 (Unc-51-like kinase involved in neurite extension) and GABARAP was associated with ULK1 in vesicles in many different classes of neurons.[38] The *c. elegans* homologue of ULK-1, Unc-51, which participates in vesicle transport, has high homology to Atg1p, a yeast serine/threonine kinase that is crucial for initiation of autophagic vacuole formation. Whether ULK-1 plays a role in autophagy is unknown.

An interesting model, also invoking autophagy, emerged in a study reporting on the mechanism of death of cultured SCG neurons habituated to survival dependent on glial-derived neurotrophic factor (GDNF).[39] When SCG neurons were isolated from neonatal rats and allowed to regenerate with NGF, they died by apoptosis upon NGF-deprivation. However, when these neurons were regenerated in the presence of GDNF, the neurons die non-apoptotically upon withdrawal of GDNF. Numerous AVs, typically with a double membrane structure, or with multilamellar membranes possibly indicating autolysosomes, appeared in the cytoplasm of neurons concomitantly with death. Interestingly, the mitochondria appeared to remain fully functional, thus divorcing the link between mitochondrial damage and activation of autophagy. Use of dominant negative forms of various caspases implicated caspase 2 and 7 in this death, so there may be a link between caspases and autophagy, though the relationship is not clear.

Clarke has been the strongest proponent of autophagy being involved in developmental cell death of certain neuron populations —defined as "type 2" cell death to distinguish it from "type 1" apoptotic death.[40] Hornung et al.[41] showed that injection of colchicine to the retina of embryonic chicks (to mimic target deprivation during development, but which also causes direct axonal injury) resulted in the accumulation of numerous vesicles in the dying isthmo-optic nucleus neurons that project to the retina. A range of organelles were found within the vesicles strengthening the notion that these are autophagic. Further recent work from this group has demonstrated autophagic arousal after administration of the glutamate mimetic N-methy-D-asparate (NMDA) to rodent hippocampal slices in vitro.[42] Within 2 hours, numerous vesicles were found in cells that already appear significantly degenerated. The region demarcated by enhanced autophagy was reminiscent of the area "cleared" in chromatolytic neurons and in other neuronal models of excitotoxicity.[43] However, only one example of an autophagosome was provided (by EM) that appears as a single membraned structure with cytoplasmic contents. The associated double membraned structure appears to be derived from the adjacent Golgi stack and does not appear to be part of this vesicle. A novel aspect of this work is its link to signalling pathways implicated in cell death. Thus, administration of a JNK-signal blocking peptide derived from the JNK binding domain of JIP1,[44] a sequence used previously to block neuronal apoptosis in response to NGF-deprivation,[45] inhibited both the increase in nuclear P-cJun associated with NMDA-induced toxicity, and the subsequent pathological changes including accumulation of AVs. In this model, as well in the model studied previously,[41] death was associated with a massive increase in endocytosis. However, Borsello et al.[46] have shown that increased endocytic activity—which might have been interpreted as a mechanism for matching the increase in autophagosome number to enable vesicle maturation into an active lysosome is not necessarily part of autophagic death and is also prevalent in a necrotic excitotoxic cell death model induced by kainic acid, which does not involve an increase in autophagy. Perhaps the rapidity of death in this necrotic model precludes activation of autophagy, just as necrosis precludes execution of apoptosis. In any case, the arguments for autophagy being a mediator of death in these models are still indirect. Clark has argued strongly for a type of death executed by autophagy,[40] and against lysosomal involvement by release of enzymes into the cytoplasm. Studies are now emerging where autophagic genes are shown to participate in apoptotic cell death.[47,48] However, there are also examples where lysosomal enzymes released from lysosomes participate in controlled forms of cell death.[49,50] Vacuolisation is also observed in the cytoplasm under both conditions but whether autophagy is involved in this type of death is still not clear. Extensive neuronal vacuolisation is observed,

for example, in the CNS neurons of a mouse in which neuropathy target esterase/swisscheese (normally inhibited by organosphosphates) has been deleted but these are unlikely to be autophagic.[51] There is no reason why both mechanisms cannot participate in cell death depending on time and stimulus.

Another form of neuronal injury in which autophagy has been implicated is that induced by metamphetamine.[52] This type of injury is inflicted specifically on dopaminergic midbrain neurons as the drug forces the intracytoplasmic accumulation of dopamine, a potentially oxidative and toxic catechol neurotransmitter. Treatment results in the accumulation of numerous vesicles described as autophagic which are correlated with loss of mitochondria and neuronal death. Typically, most of the profiles are multilamellar vesicles that could arise from lysosomes without an intermediate step of autophagy. Similar multilamaellar profiles were displayed in *C. elegans* neurons dying in response to the hyperactivation of a mechanosensory ion channel, a kind of excitotoxic death.[53] Recently this death was found to be inhibited by a series of proteases, including some cysteine proteases typically found in lysosomes.[54] Whether these become activated as a result of autophagic activity is readily testable, as autophagy has already been dissected genetically in relation to longevity in *C. elegans*.[55]

It is possible that activation of autophagy is important for production of novel forms of hybrid vesicles that then perform specific functions. The importance of interactions of autophagic vesicles with other types of vesicles, in addition to endosomes,[56,57] should not be dismissed. An interesting potential cross over point is provided by the protein Alix, which is enriched in regions of neurodegeneration induced by the mitochondrial toxin 3-nitropropionic acid and the seizure-inducing exitotoxin kainic acid,[58,59] and is possibly causal in neuronal cell death.[60] Alix induces vacuolisation which, together with other factors, creates vesicles displaying multivesicular (that is, several non-concentric vesicles) or multilamellar (concentric vesicle) organisation.[61] Its link with autophagosomes is worth exploring. AVs containing active caspases have also been described in granulovacuolar forms of degeneration[62] but again the identity of these vacuoles as autophagosomes has not been substantiated.

All the occurances of autophagy in neurons to date have been based on electron microscopy or on use of drugs that are not highly specific as autophagy inducers and inhibitors. The distinction between autophagosomes, lysosomes, endosomes, amphisomes, and their intermediates is not unequivocal. Thus, the importance of autophagy in neuronal death, survival and regeneration awaits application of molecular dissectors that can interfere with this process and distinguish between AVs and other forms of vesicles.

INSIGHTS FROM LURCHER MICE: AUTOPHAGY, NEURONAL FUNCTION AND DYSFUNCTION

One of the strongest associations between autophagy and neurodegeneration is seen in the *Lurcher* mouse, where heterozygotes with an activating mutation in the orphan glutamate receptor ($GluR\delta2^{Lc}$) develop cerebellar ataxia in the first four postnatal weeks, due to degeneration of the cerebellar cortex. This mutation proved that a molecular alteration in a neurotransmitter receptor can cause degeneration in vivo, and provided the first genetic model in which mechanisms of excitotoxic neuronal death could be intensively investigated in situ.[63] Subsequent studies revealed that lurcher Purkinje cells activate apoptotic responses in response to this mutation,[64-66] although attempts to genetically rescue Purkinje cell death in lurcher mice by blocking the apoptotic pathway were unsuccessful.[67,68] It is interesting to note that secondary death of granule cells in response to Purkinje cell loss was rescued. These data clearly indicated that, while apoptotic responses may contribute to Lurcher pathology, other mechanisms must also come into play.

To try to identify these mechanisms, Yue et al.,[23] characterized a protein complex bound to the C-terminus of the GluRδ2 receptor, identifying nPIST and Beclin1 as components of the complex. Although Beclin1 was first identified as binding to Bcl2, an inhibitor of apoptosis, it was also immediately recognized as the mammalian orthologue of Atg6 (also known as Vps30), an essential component of the yeast autophagy pathway.[69] This raised two exciting possibilities: that autophagy was the missing pathway contributing to Purkinje cell death in lurcher mice, and that the coupling of autophagy to neurotransmitter receptors plays an important role in regulating neuronal function. To investigate autophagy in the context of Purkinje cell death in lurcher mice, Yue et al.,[23] first showed that a fusion of Beclin1 and green fluorescent protein (Beclin1-GFP) could be used as a convenient visual assay for autophagy. They then used this assay to demonstrate that nPIST and Beclin1 could synergize to induce autophagy, that autophagy is induced in heterologous cells transfected with $GluR\delta2^{Lc}$ but not wild type GluRδ2, and that the death of cells transfected with the mutant receptor could be partially rescued by pharmacologic inhibition of autophagy with 3-methyladenine. However, there is the caveat mentioned earlier that this compound also inhibits death signalling via JNK and p38, and mitochondrial dysfunction.[27] Electron microscopic examination of dying lurcher Purkinje cells in vivo revealed massive induction of autophagy. These data demonstrated a biochemical link between the neurotransmitter receptor GluRδ2 and autophagy, and suggested that constitutive activation of autophagy in response to the mutant receptor could lead to cell death. The model that emerged from these studies is that both apoptosis and autophagy can contribute to excitotoxic cell death in vivo, and that Beclin1 is the key molecule orchestrating the relative contributions of these two pathways.

The activation of autophagy in response to signalling through the $GluR\delta2^{Lc}$ receptor raises important issues for models of excitotoxic cell death. For example, one would like to know the relative contributions of ion influx and depolarization of the cell membrane, and excess signalling through receptor-bound complexes to excitotoxic cell death. Insight into this issue in lurcher mice has been obtained through studies of the $GluR\delta2^{Lc}$ allele bred onto a hotfoot (a null allele of GluRδ2) background.[70] In these mice, Purkinje cell death is accelerated, occurring prior to the stage at which depolarization can be measured in electrophysiologic recordings. Nevertheless, electron microscopic analysis of these cells clearly demonstrates that autophagy is activated. These data provide strong evidence that massive ion flux into the cell and the consequent depolarization of the cell membrane are not critical for $GluR\delta2^{Lc}$ mediated cell death in vivo. The evidence collected from studies of lurcher mice, therefore, strongly suggest that constitutive activation of receptor-mediated cell signalling, and the consequent induction of downstream pathways including autophagy, are the critical events that underlie neurodegeneration. While these data strongly couple autophagy to cell death, a direct causal role for autophagy in the cell death execution has yet to be definitively proven in vivo.[71] The fact that the coupling of autophagy to GluRδ2 (which is expressed principally in cerebellar Purkinje cells) occurs through nPIST and Beclin1 (which are very widely expressed in the nervous system) suggests that this pathway is regulated by a variety of other neurotransmitter receptors and their

ligands. Since synaptic abnormalities have been associated with a wide variety of neurological disorders, the activation of autophagy in these diseases might result from aberrant synaptic signalling events. Although the studies of lurcher mice strongly suggest that excess activation of autophagy can contribute to neurodegeneration in response to excitotoxic stimuli, the activation of autophagy via synaptic stress may in many cases first serve a neuro-protective role. Our understanding of the positive and negative consequences of autophagy in the context of neurological disease will be greatly advanced by genetic dissection of this pathway in other animal models of neurological disease.

The most important discovery in these studies of lurcher mice is the simple fact that autophagy is directly regulated by a neurotransmitter receptor. Although this has important implications in a disease context and in the context of excitotoxicity, the linking of autophagy and neurotransmitter activity is also likely to be of fundamental importance to normal synaptic function. For example, the GABA receptor associated protein GABARAP[72] has been shown to be important for trafficking of GABA receptors within the cells.[73] Given this result, and the coupling of autophagy to the GluRδ2 receptor, one might imagine a more general role for autophagy in trafficking and turnover of neurotransmitter receptors at the synapse. This is consistent with studies of Purkinje cell synapses in mice carrying null mutations of GluRδ2 receptor, where a variety of synaptic abnormalities have been observed.[74] The contributions of autophagic mechanisms to fundamental mechanisms of synaptic function, including protein trafficking and turnover at the synapse and synaptic remodeling are important areas for future investigation.

ALZHEIMER'S DISEASE AND AUTOPHAGY

Alzheimer's disease (AD), the most prevalent human neurodegenerative disorder, involves a progressive loss of neurons leading to profoundly impaired memory and declines in other intellectual functions. AD is defined by two hallmark neuropathological lesions, the intra-neuronal neurofibrillary tangle containing aggregated forms of the microtubule-associated protein, tau, and the neuritic plaque, mainly composed of extracellular aggregates of β-amyloid peptide (Aβ) in association with degenerating dendrites or axons.[75] Preceding cell death, the synaptic terminals, axons and dendrites of neurons undergo a process of degeneration and abortive regeneration over many years,[76-80] which creates a highly characteristic pattern of grossly enlarged, "dystrophic" neurites.[81-83] Beginning at the earliest stages of this process, the lysosomal system of neurons in affected brain regions is mobilized[84]: lysosomes proliferate, the expression of lysosome-related components increases, and the trafficking of cathepsins to early endosomes is accentuated.[84-88] As neurons become more metabolically compromised, lysosomes and other hydrolase-containing compartments progressively accumulate. Indeed, cathepsin D mRNA production remains highly elevated in single neurofibrillary tangle-bearing neurons even though most transcripts unrelated to the lysosomal system decline or remain unchanged.[89] Increased or altered activity of the endocytic pathway has been considered one possible stimulus for lysosomal system mobilization in AD[85,90] but macroautophagy is emerging as a key driving force for this and other features of AD neuropathology.

Although rarely in evidence in the normal mature brain, macroautophagy is induced in vulnerable populations of neurons at early stages of AD and in a transgenic mouse model of AD (PS/APP), in which human mutant presenilin-1 (PS-1) and the Swedish variant of the amyloid precursor protein (APP) are overexpressed.[91-93] Mutations in PS-1 and APP cause autosomal dominant, early-onset AD. In both conditions, autophagosomes and other AVs proliferate in dendrites and the levels of LC3-II, a specific marker of autophagosome formation, are elevated.[91-93] LC3 in affected brain regions of PS/APP mice translocates to vesicles, reflecting the conversion of LC3-I to LC3-II, and both LC3 isoforms redistribute to dendrites from a mainly perikaryal location. This process begins at young ages before β-amyloid pathology develops in these mice indicating that macroautophagy induction is an early response in the disease and not solely a consequence of amyloid deposition. Autophagy-related pathology in AD and PS/APP mouse brains progresses as autophagosomes and other AV subtypes, together with hydrolase-positive dense bodies,[94-96] accumulate in large numbers in grossly distended dystrophic neurites, becoming the principal organelle in these structures. Although AV accumulations are not specific to the degenerative phenomena of AD,[23,97-99] autophagic-lysosomal pathology in the brain is considerably more widespread and robust in AD than in other adult-onset neurodegenerative diseases.[100] Specifically, the extensive neuritic dystrophy[101,102] and the characteristic gross distension of these neurites in AD are not typical in other neurodegenerative diseases lacking β-amyloid.[103] Moreover, the near replacement of cytoplasmic contents in the dystrophic neurites, together with the high incidence of AVs in less obviously affected neurites, constitute a uniquely large "burden" of autophagy-related compartments in the AD brain.

Macroautophagy is normally active within the growing (regenerating) neurites of cultured neurons.[104-106] An early induction of macroautophagy in AD might, therefore, be expected to support the organelle and protein turnover associated with cycles of neurite degeneration and regeneration[107-109] in affected neurons, which also revert to a more immature developmental state in AD.[110,111] Macroautophagy may also protect neurons in other ways.[92,54] Activated caspases are detected in the AVs of PS/APP mice and in the granulovacuolar degeneration (GVDs), which are characteristic vacuoles of suspected autophagic origin in hippocampal neurons of AD brain,[62] raising the possibility that macroautophagy might protect neurons by turning over apoptosis-inducing factors, including damaged mitochondria.[112-115]

Ultimately, macroautophagy function in dendrites seems gradually to become impaired during the course of AD. In normal growing (regenerating) neurites of cultured neurons, immature AVs are transported retrogradely toward the cell body where they encounter, and fuse with, acidified hydrolase-containing compartments.[104,105] AV maturation to autophagolysosomes is efficient under these conditions, resulting in the rapid degradation of AV contents, with minimal evidence of intermediate autophagic compartments. The enormous buildup of both early and late AVs within dystrophic/degenerating neurites in AD brain, however, suggests that the transport of AVs may be impeded or that autophagy locally within the dystrophic segments is particularly robust. In addition, the coexistence of both immature and acid-hydrolase-containing compartments in substantial numbers in the dystrophic neurites suggests that immature AVs have access to hydrolase-containing compartments but may less efficiently carry out the final stages of degradation. This possibility is further suggested by observations on the forms of early-onset familial AD (FAD) caused by mutations of presenilin-1 (PS1).[116-118] PS1 mutations not only induce more severe amyloid pathology and neurodegeneration in PS-FAD and in mouse models but also potentiate lysosomal system pathology.[119] Fibroblasts from patients with PS-FAD

also abnormally accumulate AVs, especially under conditions that stimulate autophagy. Surprisingly, however, the turnover of long-lived proteins by autophagy in these fibroblasts is markedly reduced. A direct role for mutant PS1 in this defect is supported by observations that autophagy-mediated protein degradation is nearly eliminated in blastocysts from mice lacking PS1 and PS2 genes but rescued by reintroducing PS1.[120] Combined with evidence that PS1 and the γ-secretase complex are enriched in AVs,[93,121] these studies support the hypothesis that PS plays a key role in autophagy and that FAD-linked PS1 mutations have a loss of function effect on macroautophagy leading to AV accumulation and impaired protein turnover.

Because proteasome activity declines in aging and is further impaired in AD,[122,123] a loss of autophagy function, if it develops in AD, leaves neurons with no competent mechanism to remove abnormal and potentially toxic proteins. Autophagy does not prevent, and may even promote, the overexpression of cathepsins, which continue to accumulate progressively in AD. The accumulated hydrolase-containing AVs in dystrophic neurites represent a large local reservoir of proteases that, if released, have the potential to initiate or mediate aspects of apoptotic and necrotic cell death in various pathological settings.[84,112,115,124-127]

The relationship of macroautophagy to AD pathogenesis is not limited to neurodegeneration: an intimate connection to β-amyloidogenesis also seems likely. AVs isolated from several different sources are enriched in components required for Aβ generation and macroautophagy is a significant pathway for Aβ peptide generation.[93,121] AVs contain immunoreactive Aβ peptide and are enriched in its immediate precursor. They are also highly enriched in components of the γ-secretase complex and exhibit high presenilin-dependent γ-secretase activity.[93,121] Senile plaques in AD and PS/APP mouse brains are also strongly immunoreactive for these components and presenilin-1 localizes prominently to AVs in dystrophic neurites.[93] Presenilin and nicastrin, another component of the γ-secretase complex, have been reported to be enriched in lysosomes[128] although AVs, which were not distinguished from lysosomes in these earlier studies, are a more concentrated localization of these γ-secretase components. Macroautophagy-mediated Aβ generation has been demonstrated by evidence that Aβ production rises when autophagy and AV proliferation are stimulated and falls when autophagy is inhibited by blocking either of two independent signalling pathways converging on mTOR kinase.[93] Interestingly, inclusion body myositis, the only known condition in which β-amyloid deposits outside the nervous system,[129] involves the accumulation of autophagy-related "rimmed" vacuoles containing APP, Aβ, BACE and presenilin.[130]

Aβ peptide is believed to be generated at several sites within neurons, including endosomes, Golgi, and ER[86,88,128,131] and each of these organelles could contribute to the involvement of autophagy in Aβ generation. Endocytic and autophagic pathways communicate extensively and both Golgi and ER are turned over by autophagy.[132] In normal brain, autophagy may play a relatively minor role in constitutive Aβ generation; however, in AD, where AVs accumulate in large numbers and persist in dystrophic neurites, autophagy can contribute substantially to overall β-amyloidogenesis, a process promoted in the mildly acidic pH environment of lysosome-related organelles.[133] In theory, AV accumulation associated with any significant neuritic injury could stimulate local Aβ production, as seen for example, in traumatic brain injury.[134] The chronic and extensive remodeling of neurites in AD, however, represents a particularly favorable condition for Aβ generation by the autophagic pathway.

As a pathogenically important pathway for Aβ generation and a mediator of both cell survival and degenerative phenomena,[40,84,135] macroautophagy, therefore, represents a new direction for investigations into the pathogenesis and possible therapy of AD.

AUTOPHAGY AND PARKINSON'S DISEASE

Parkinson's disease (PD) is characterized neuropathologically by selective neuronal degeneration, primarily in dopaminergic neurons of substantia nigra pars compacta (SNpc) and other brainstem nuclei, and the accumulation of filamentous intracytoplasmic inclusions, termed Lewy bodies (LBs). Ubiquitin and α-synuclein constitute major components of LBs, and recent studies using α-synuclein antibodies have revealed that the extent of neuropathology in PD is quite widespread.[136,137] α-Synuclein is a protein of unclear function that localizes predominantly to presynaptic terminals in the mature CNS.[138] While the genetic factors underlying most forms of late-onset, apparently sporadic PD are poorly understood, missense or duplication/triplication mutations in α-synuclein lead to PD in rare familial cases that follow a dominant pattern of inheritance, indicating a gain-of-function effect.[138] Other genetic defects have also been detected in rare families with familial PD,[139] although the majority of cases of PD are sporadic, with no clear genetic basis.

There have been few pathological studies addressing the issue of autophagy in Parkinson's disease. Forno and Norville,[140] when assessing sympathetic ganglion LBs in PD, remarked on the rather vesicular nature of these inclusions, suggesting the participation of the autophagic/lysosomal pathway in the formation or dissolution of these inclusions. Anglade et al.[97] reported autophagosome-like structures in degenerating neurons in the SNpc of PD patients. In fact, the images that were shown made a more convincing case for autophagy compared to apoptosis.[97] More recently, AVs have been found to engulf mitochondria in PD substantia nigra.[141] Given the abundant genetic, biochemical and pathological data implicating mitochondrial dysfunction in PD,[139] such dysfunction may serve as the trigger for the activation of macroautophagy, as has been shown in other systems.[31]

Neuromelanin is a pigment that accumulates with age selectively within certain populations of catecholaminergic neurons, including those of the SNpc. Sulzer et al.[142] reported that neuromelanin granules are actually autophagosomes, as they are delimited by double membranes. They have hypothesized that such granules sequester dopamine and its quinone metabolites and thus prevent their potentially lethal accumulation in the cytosol. Consistent with this idea, manipulations that led to a decrease of intracellular dopamine prevented the formation of such granules in an experimental neuronal cell culture system, where granules were induced by application of L-Dopa, the precursor of dopamine.[142] It appears therefore that macroautophagy is quite active even in normal SNpc, presumably serving a protective function. The possibility that this attempt at sequestration may ultimately lead to problems of its own was suggested by the finding that PC12 cells bearing such neuromelanin granules showed a less robust response to Nerve Growth Factor (NGF) application.[142] The reason why neuromelanin granules arrest at the phase of autophagosomes and do not convert to mature lysosomes is unclear.

There is evidence for activation of macroautophagy in some cellular models related to PD. Overexpression of murine α-synuclein in a neuronal cell line induced autophagy, without cell death.[143] Stefanis et al.[144] showed that stable overexpression of mutant A53T, but not WT, α-synuclein in PC12 cells, led to the marked induction of macroautophagy and cell death without the morphological or

biochemical features of apoptosis. Cells expressing the mutant form expressed a wide range of other abnormalities, including proteasomal and lysosomal dysfunction. Stefanis et al.[144] hypothesized that such abnormalities in the main cellular degradation systems may be responsible for the induction of macroautophagy. Recent evidence provides further support for this hypothesis. PC12 cells expressing mutant α-synucleins are deficient in the degradation of protein substrates through the chaperone-mediated autophagy (CMA) pathway.[145] The upregulation of macroautophagy that occurs in PC12 cells expressing mutant α-synucleins appears therefore to be a compensatory response, following the primary defect at the level of CMA.[145] The primary aberrant effect appears to be an inappropriately tight binding of mutant α-synucleins to lamp2a, the receptor component of CMA. In this fashion, mutant α-synucleins do not permit the binding and uptake of the normal endogenous substrates of CMA.[145] Thus, in this cellular model, macroautophagy is a compensatory response of the cell attempting to cope with constraints imposed on its ability to degrade proteins, and not a primary mechanism of cell death induction. The molecular underpinnings of such compensatory responses are not yet understood. Notably, and despite macroautophagy activation, overall levels of lysosomal degradation were diminished in the lines expressing mutant α-synucleins. This is presumably due to the fact that CMA makes a major contribution to lysosomal degradation in the cells examined, especially under conditions of serum depletion.[145] When CMA is inhibited in the mutant α-synuclein lines, the compensatory activation of macroautophagy is presumably not enough to restore normal levels of lysosome-dependent protein degradation.

It has also been hypothesized that PD may be caused, at least in part, by dysfunction of the other major intracellular degradation system, the ubiquitin-dependent proteasomal system.[146] It has recently been demonstrated that neuronal cell lines treated with low doses of proteasomal inhibitors activate macroautophagy as a compensatory response.[147] Similar findings have been reported earlier in HeLa cells acutely treated with proteasomal inhibitors.[148] In the first such study in primary neurons, Rideout et al.[149] observed marked induction of macroautophagy and activation of the lysosomal pathway in cultured cortical neurons treated with proteasomal inhibitors. Furthermore, pharmacological modulation of macroautophagy led to alterations in the number of LB-like ubiquitinated inclusions formed in this model, suggesting that macroautophagy may be responsible for inclusion dissolution.[149]

A related issue that is quite controversial is the degradation of α-synuclein itself. Initial studies showed that α-synuclein, in some cases in a polyubiquitinated form, accumulated in cells upon proteasomal inhibition, suggesting that the proteasome was responsible for α-synuclein degradation through the ubiquitin-dependent pathway.[150-152] A subsequent study, based on a purified system, showed that α-synuclein, as would be predicted from its natively unfolded state, does not need to be ubiquitinated to be degraded by the proteasome.[153] To complicate matters, other studies have failed to replicate the finding of accumulation of endogenous or overexpressed α-synuclein with proteasomal inhibition.[154-157] Some of these studies in particular were performed with endogenous α-synuclein in PC12 cells or cultured cortical neurons,[155,156] and thus may reflect more closely the normal turnover of the protein. While some of the earlier studies may have suffered from limitations due to the artificial transient nature of α-synuclein overexpression, the presence of epitope tags, and the expression in a non-neuronal environment, other studies have observed that proteasome inhibition impairs α-synuclein clearance in both differentiated and undifferentiated PC12 cells.[158] One reason for these apparent discrepancies is that α-synuclein may be processed via a diversity of pathways and that a small change in turnover resulting from proteasome inhibition (which may not be a major degradation route) may not rapidly or overtly translate into alterations in steady-state levels. There is now considerable evidence that α-synuclein levels can accumulate with lysosomal inhibition, although the exact circumstances (cellular milieu, species of α-synuclein, class of lysosomal inhibitor) differ across studies.[145,157-160] In the most physiological system examined so far, that of cultured post-natal rat dopaminergic neurons, endogenous α-synuclein turnover was significantly increased only by the general lysosomal inhibitor ammonium chloride, but not by the macroautophagy inhibitor 3-methyladenine. Consistent with a minor role of the proteasomes in α-synuclein degradation, application of epoxomicin, the selective proteasomal inhibitor, provided only a very small increase in the half-life of α-synuclein in this setting. In fact, α-synuclein contains a pentapeptide motif that targets it to the pathway of CMA. Analysis in an in vitro system of purified lysosomes has confirmed that α-synuclein can be degraded by CMA. Coupled with the cellular data, which indicate that α-synuclein is degraded by a lysosomal pathway that is not macroautophagy, CMA appears to be the major rate-limiting pathway used for wild-type α-synuclein degradation in a neuronal cell context.[145] Furthermore, the mutants A53T and A30P are deficient in CMA-dependent degradation, a fact that leads to their accumulation, but also to the aberrant effects on the CMA pathway mentioned earlier.[145] Interestingly, the A53T and A30P mutants show a significant dependency on macroautophagy for their clearance.[145,158] The clearance of these proteins is delayed with inhibitors of macroautophagy and enhanced by rapamycin (which induces macroautophagy). By contrast, as mentioned, wild-type α-synuclein clearance is not obviously affected by macroautophagy inhibitors.[145,158] Interestingly, the clearance of wild-type α-synuclein is enhanced by rapamycin in both differentiated and undifferentiated PC12 cells.[158] While macroautophagy under normal circumstances is not rate-limiting for wild-type α-synuclein clearance (probably because CMA and maybe the proteasome are the major pathways under basal conditions), it is possible that when macroautophagy is upregulated, it can provide an additional significant clearance route for this protein. Consistent with this, endogenous α-synuclein has been localized to autophagosomes in certain situations, for example in brains of mice that lack PS1.[26]

Thus, the pathways used for α-synuclein degradation may vary depending on a number of factors, such as the neuronal cell type and the metabolic state. In addition to the proteasome and the lysosomes, other proteolytic systems, such as calpains[161] or callikrein-6 (neurosin)[162] may also play a role in α-synuclein cleavage and degradation. It would also be interesting to study the role of macroautophagy in α-synuclein clearance.

In conclusion, macroautophagy appears to occur under physiologic conditions in SNpc, and to be further induced in PD. It is likely to represent a compensatory response to the dysfunction of other intracellular degradation pathways, mitochondrial dysfunction or the accumulation of cytosolic dopamine. The attempt of the neurons to activate macroautophagy may eventually backfire, leading to further cellular dysfunction and autophagic death. Degradation of α-synuclein in lysosomes may be closely related to its aberrant effects. In particular, disruption of CMA may be a primary pathogenic effect of mutant α-synucleins. While mutant forms of α-synuclein are very rare, it may be important to consider if there is dysfunction of CMA in sporadic PD.

PRION DISEASES AND AUTOPHAGY

Human transmissible spongiform encephalopathies, also known as prion diseases, include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, Creutzfeldt- Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS), fatal familial insomnia (FFI), kuru and most recently variant CJD (vCJD) in humans. The molecular basis of prion disease is the post-translational conversion of a normal host-encoded, glycosylphosphatidylinositol (GPI)-anchored glycoprotein, the cellular prion protein (PrP^{C}), to an abnormal isoform, designated PrP^{Sc}. This transition confers PrP^{Sc} with a partial resistance to proteolytic degradation and detergent insolubility.[163] Consistent with the idea of an altered conformation is the finding that β-sheet content in PrP^{Sc} is higher than in PrP^{C}.[164]

The neuropathology of prion diseases includes the accumulation of an abnormal protein PrP^{Sc} in the brain, neuronal vacuolation (spongiosis), neuronal loss, proliferation of astrocytes and microglia, and deposition of amyloid plaques.[165-167] Studies indicate a role for autophagy in prion disease pathogenesis. In an experimental model of CJD in mice, giant AVs formed in cortical neurons.[160] The AV appeared at the end of the incubation period (4–6 months postinoculation), together with spongiform changes and clinical symptoms.[168] The formation of giant AV was also found in neurons in experimental scrapie in hamsters.[169] The ultrastructural features of autophagy in experimental scrapie and CJD in rodent brain are similar[170] and include membrane sequestration of neuronal cytoplasm, membrane proliferation, and the formation of AVs of various sizes. An important observation in the animal models has been the presence of AVs in all parts of the neuron including synaptic endings.[171] Recent electron microscopic analysis of human brain biopsies from patients with CJD, GSS, and FFI revealed predominant changes in axon terminals that included the presence of AVs and features consistent with axonal degeneration.[172] These findings suggest that synaptic dysfunction associated with autophagic dysregulation could precede other clinical and pathological features of these disorders. While there is extensive evidence for increased autophagosome numbers in prion disease brain tissue, it is unclear if the altered PrP conformation enhances autophagic activity or delays autophagosome clearance and if this is in any way related to the ensuing neuronal death.

POLYGLUTAMINE EXPANSION DISEASES AND AUTOPHAGY

Polyglutamine diseases are a group of inherited neurodegenerative disorders caused by CAG trinucleotide repeat expansion mutations that are translated into abnormally long polyglutamine tracts in the mutant proteins. Huntington's disease (HD) is the most common of these diseases, which also include spinocerebellar ataxias types (SCA) 1, 2, 3, 6, 7 and 17, spinobulbar muscular atrophy (SBMA) and dentatorubral-pallidoluysian atrophy (DRPLA). Studies in humans and in mouse models suggest that polyglutamine expansion mutations are predominantly associated with a deleterious gain-of-function. One of the pathological hallmarks of this class of diseases is the formation of intracellular aggregates (also known as inclusions), which are formed by the mutant protein and also include other proteins. The roles of these inclusions are controversial (reviewed in ref. 173).

Another feature of certain polyglutamine diseases is the presence of increased numbers of autophagosomes in cell models and in vivo. In the brains of HD patients, immunoreactive huntingtin (HD gene product; htt) was found in cytoplasmic granules that were identified at the electron microscopic level to be multivesicular bodies. These organelles are involved in protein degradation.[174] This observation supported the idea that the endosomal/lysosomal pathway might be involved in htt degradation. Indeed, exogenous expression of full-length or truncated wild-type or mutant htt in clonal striatal cells results in the association of htt with tubulovesicular organelles and with autophagosomes.[98] The ultrastructure of the htt-associated autophagosomes seen in vitro is diverse and includes both early autophagosomes with double-membranes and late autophagosomes. Deposition of htt occurs within the lumen and on the limiting membranes of early and late autophagosomes. Autophagosomes bound by truncated or full-length htt that are distributed throughout the cytoplasm coalesce in perinuclear regions to form htt bodies.[99] The mutant htt bodies contain fibrillar and soluble oligomeric forms of mutant htt. The polyglutamine expansion in htt significantly increases the frequency of cells with htt-associated autophagosomes. Primary striatal neurons from HD transgenic mice expressing truncated mutant htt showed a marked increase in autophagosome/lysosomal bodies following dopamine-stimulated oxidative stress.[175] In addition, HD patient lymphoblasts appear to form more autophagosomes compared to control lymphoblasts.[176] There is some evidence for enhanced autophagy occurring with expression of other polyglutamine disease proteins. Mice expressing mutant ataxin 1 (mutated in SCA1) show formation of degradative vacuoles in degenerating Purkinje cell dendrites.[177] HEK-293 cells exogenously expressing truncated mutant androgen receptor (with a polyglutamine expansion as in SBMA) show patches of immunoreactive mutant protein associated with large-double membraned structures and with lysosomes.[178]

One key question is whether the increased autophagosome numbers in HD and related diseases are due to increased autophagosome synthesis, or decreased autophagosome-lysosome fusion. It is possible that similar mechanisms may exist between different polyglutamine expansion diseases, given their shared mutations.[179] Another important issue is if this phenomenon is a protective response, a necessary part of a death pathway, or an epiphenomenon.

In cell models of HD and related polyglutamine diseases (like SCA1 and DRPLA), increased autophagy (assessed with LC3-II blots and LC3 immunocytochemistry) appeared to be specific to cells expressing mutant constructs that also had aggregates. The increased autophagic activity in these contexts appeared to be due to the sequestration and functional inactivation of mTOR (a negative regulator of autophagy) by the polyglutamine aggregates. mTOR inactivation in cells with aggregates was also consistent with data from the brains of an HD mouse model. This may be a mechanism accounting for enhanced autophagy in diseases other than HD as mTOR sequestration was seen in the aggregates in brains of patients with a number of different polyglutamine expansion diseases.[179] Thus, increased autophagy in polyglutamine diseases may be due to increased autophagosome production, due to inhibition of mTOR, which negatively regulates an early stage in this pathway.

Since polyglutamine mutations act primarily by conferring toxic novel properties on the mutant protein, it is important to understand how they are cleared in cells. This is particularly relevant for post-mitotic neurons, where the proteins cannot be diluted by cell division. While the proteasome can process soluble monomeric forms of huntingtin, its narrow pore precludes proteasomal clearance of oligomeric and aggregated species. Furthermore, the proteasome cannot cleave within the polyglutamine tract.[180] Thus, the proteasomes effectively remove the sequences flanking the polyglutamine stretch and leave an expanded polyglutamine stretch with almost no flanking

sequences. This may enhance toxicity/misfolding—previous studies have shown that toxicity/aggregation is reduced when more sequence flanks the polyglutamine expansion (e.g., in huntingtin).[181]

Autophagy represents an alternative route for clearance of these aggregate-prone polyglutamine-expanded proteins. Chemical inhibition of the autophagy-lysosomal pathway at the autophagosome formation or autophagosome-lysosome fusion stages inhibited the clearance of mutant huntingtin fragment and was associated with increased soluble mutant protein levels, and enhanced aggregate formation and toxicity in cell models.[182] The dependence of huntingtin clearance on autophagy was not only a feature of short fragments of the mutant protein but also for longer products with the expanded repeats.[99] Consistent with these data, mutant proteins with polyglutamine expansions (from Huntington's disease, spinocerebellar ataxia type 3 and dentatorubral pallidoluysian atrophy) are seen in lysosomes in patient brains.[183] These data suggest that the dependence on autophagy is greatest for the most aggregate-prone species. In other words, the wild-type fragments of huntingtin show minimal accumulation when autophagy is blocked, in contrast to the mutant constructs. This phenomenon is likely to be because the aggregate-prone species are less efficiently cleared by the proteasome—autophagy is a "default" pathway. While the size of typical mammalian autophagosomes (~1.5 μm) suggests that they cannot clear large aggregates visible by light microscopy, they may be able to clear monomeric forms or small microaggregates/oligomers.

Enhancing the clearance of toxic, aggregate-prone, mutant proteins by autophagy may be a potential therapeutic avenue for diseases like HD. Rapamycin, which activates this pathway, enhanced the clearance of mutant huntingtin fragment, and reduced aggregate formation and toxicity in cell models.[182] Rapamycin also protected against neurodegeneration in a *Drosophila* HD model and a rapamycin analogue, CCI-779, improved various HD-associated behavioral tasks and decreased aggregate formation in a transgenic HD mouse model.[179]

In summary, enhanced autophagy is seen in HD and related polyglutamine diseases. This phenomenon appears to be at least in part due to the ability of aggregated huntingtin to sequester and inactivate mTOR. While aggregates may result in both deleterious and protective consequences, the sequestration of mTOR appears to represent a protective response, as enhancing autophagy with rapamycin protects against polyglutamine toxicity in cell and animal models.

CONCLUSION

For most scenarios, the key questions outlined in the introduction remain as uncertainties. To reiterate: Are increased autophagosome numbers due to increased autophagy or decreased autophgosome-lysosome clearance and is increased autophagy good, bad or an epiphenomenon? A related question is how autophagy interacts with and contributes to different cell death pathways. While data in cell and animal models suggest that autophagy induction may be beneficial in HD, this needs further development and refinement before embarking on long-term human trials. Also, it is worth considering if this strategy may be beneficial to other diseases caused by toxic, aggregate-prone proteins. It is likely that the answers to these questions vary in different contexts. It is also interesting to consider that the roles of autophagy and even possibly the machinery regulating the process may be different in response to different stimuli, or in the central nervous system compared to peripheral tissues (like the liver). While there has been great progress in understanding the biology of autophagy, particularly in yeast, many aspects of mammalian autophagy regulation remain poorly understood, limiting the tools available to address these issues. Thus, a major challenge remains to define and characterize the machinery regulating mammalian autophagy.

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