Mini-Review

Autophagy

Cerebral Home Cooking

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Cell survival depends on a complex interplay of processes including homeostatic pathways and cytoprotective mechanisms. Autophagy is a physiological process involved in the basal turnover of long-lived proteins and organelles and also comprises an integral part of the cellular stress response. The significance of autophagy in regulating neural cell fate has become increasingly recognized and agents targeting autophagy are of increasing therapeutic interest. This review summarizes the recent expansion of our understanding of the scope of this physiological process. (Am J Pathol 2010, 176:1065–1071; DOI: 10.2353/ajpath.2010.090850)

The mature mammalian neuron, as a long-lived post-mitotic cell, has been a popular candidate for Orgel's Error Catastrophe theory of aging, which proposes that an accumulation of transcriptional errors can trigger age-related degenerative changes and/or compromise cell survival.¹ Resultant accumulation of mutant proteins, reactive oxygen species, and/or damaged organelles leads to subsequent neuronal dysfunction and death. This might explain the predisposition of neurons to undergo age-related degenerative changes. On the other hand, this also underscores the need for a stringent quality control mechanism to eliminate abnormal or dysfunctional proteins so as to maintain neuronal function and viability. In eukaryotic organisms, this important task is performed by two main systems—the ubiquitin–proteasome system and the autophagy–lysosomal pathway. This minireview will focus on the role of autophagy in maintenance of homeostasis and regulation of neuronal function and survival.

Autophagy, a ubiquitous process, is a recycling mechanism whereby cellular content including organelles are engulfed in membrane-bound vacuoles (autophagosomes) and delivered to lysosomes for subsequent

degradation by the cache of proteolytic enzymes in the lysosomal compartment. Autophagy may be divided into three main subtypes: microautophagy, macroautophagy, and chaperone-mediated autophagy. Macroautophagy (hereafter referred to as autophagy) is the most prevalent form of autophagy occurring in mammalian cells and has received considerable recent attention. Traditionally, autophagy has been considered to be a cellular response to nutrient stress, which involves recycling of cellular content and subsequent replenishment of cellular energy levels. However, in the context of the nervous system where conventionally defined nutrient stress is rarely encountered, the physiological significance of autophagy is by no means limited to “self-eating” and has expanded dramatically to include functions in cellular housekeeping and maintenance of genomic stability, as well as development.

Basic Mechanism

Autophagy is a highly regulated process that may be broken down into several discrete steps: induction, vesicle nucleation, elongation, vesicle docking and fusion with lysosomes, and degradation of vesicle contents (Figure 1). It is controlled by a host of autophagy-related genes (*Atg*).² Over 30 *Atg* proteins have been identified in yeast, and around 10 of them have been found to have homologs in mammalian cells.

Upstream of the *Atg* proteins is the major regulatory kinase TOR (target of rapamycin), which responds to nutrient rich conditions by shutting off autophagy by preventing the *Atg1-Atg13* interaction.³ TOR activation, and hence autophagy inhibition, is effected by the GTPase Rheb and by the class I phosphoinositide 3-kinase (PI 3-K) through activation of Akt/protein kinase B and 3-phosphoinositide-dependent protein kinase 1 (PDK1) in response to insulin-like or other growth factor signals.⁴

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Figure 1. Schematic illustration of the multi-stage autophagic process. **A:** Initiation of autophagy is under the control of the master regulator TOR, which negatively modulates autophagy by phosphorylation of downstream Atg proteins. Regulators of TOR activation/inactivation include the insulin signaling pathway, p53, and the Rheb GTPase. **B:** Membrane nucleation involves the formation of the Vps34 (Class III PI 3K) complex. This step is negatively regulated by Bcl2 family members. **C:** Elongation of the membrane is mediated by two ubiquitin-like systems resulting in the formation of the autophagosome. **D:** The final stage of autophagy involves the docking and fusion of autophagosomes with lysosomes, with subsequent degradation of vesicular content by lysosomal proteases.

On the other hand, autophagy induction is mediated by negative regulators (direct/indirect) of TOR such as phosphatase and tensin homolog (PTEN; a 3' phosphoinositide phosphatase), sensors for low energy/nutrient states such as 5'-AMP-activated protein kinase (AMPK), and the eukaryotic initiation factor 2 α .^{4,5} The p53 signaling machinery has also been shown to be part of the regulation process via its activation of AMPK.⁶

Vesicle nucleation is the next step in the autophagic process and involves activation of Vps34 (a class III PI 3-K), which occurs via the formation of a complex involving beclin-1 (the mammalian ortholog of Atg6), UV irradiation resistance-associated tumor suppressor gene (UVRAG), Ambra1 (activating molecule in beclin-regulated autophagy), and Vps15 (a myristylated kinase). This step is negatively regulated by Bcl-2 family members such as Bcl-2 and Bcl-X_L, which by a direct physical association with beclin-1 prevent complex formation.⁷ However, another Bcl-2 family member, BNIP3 has been shown to re-

lieve beclin-1 from this negative interaction by competing for binding to Bcl-2.⁸ Recently, the mitogen activated protein kinase/extracellular signal-regulated kinase pathway has been shown to positively affect beclin-1 levels.⁹

The vesicle elongation step involves two ubiquitin-like conjugation systems—the first involves the formation of the Atg5–Atg12 complex by the actions of the E1-like enzyme, Atg7 and the E2-like enzyme, Atg10. The second involves the conversion of the soluble form of LC3/Atg8, also known as LC3-I, to the autophagosomal membrane-associated form LC3-II following conjugation of phosphatidylethanolamine (PE) through the actions of Atg4 (a protease), Atg7, and the E2-like enzyme, Atg3.¹⁰

Finally, autophagosomes fuse with lysosomes wherein their contents are degraded by the proteolytic activity of lysosomal enzymes. The molecular machinery of autophagy has still not been completely defined and remains the focus of intense inquiry.

Detection of Autophagy

Much of the progress in our knowledge about autophagic mechanisms has been aided by an array of methods to assess autophagy and various molecular and pharmacological tools that affect different steps of this pathway. Autophagy can be monitored by several assays—some measuring steady state levels and others following flux through the pathway.¹¹ Methods measuring steady state levels depend on the detection of autophagosomes. These include electron microscopic analysis of cells or tissues and the detection of LC3-II (via Western blotting as well as fluorescence microscopy). However, increased levels of autophagosomes do not necessarily reflect increased autophagy induction and may be a reflection of blocked autophagosome fusion or degradation. These alternative explanations for autophagic vacuole (AV) accumulation underscore the importance of measuring autophagic flux levels through protein degradation assays, LC3-II turnover, and lysosome–autophagosome colocalization.¹¹

Assessment of the role of autophagy is performed using pharmacological agents that induce autophagy (eg, rapamycin, inhibitor of mTOR) as well as those that inhibit either induction (eg, 3-methyladenine, phosphoinositide 3-kinase inhibitor) or degradation (eg, bafilomycin, inhibitor of lysosomal vacuolar ATPase) or through genetic approaches using targeted gene disruptions or RNA interference to knockdown critical components of the autophagic machinery. Experiments using pharmacological agents suffer from the limitation that these agents may have effects on nonautophagic pathways and hence, genetic approaches that target specific *Atg* genes are considered more rigorous. While knockdown approaches targeting *Atg* genes may be considered more autophagy-specific, the possibility that *Atg* proteins may cross talk with other pathways cannot be ruled out, and in fact *Atg5* has been shown to have “apoptotic connections.”¹²

Constitutive/Basal Autophagy

A recent study of primary cortical neurons has demonstrated that low levels of observed AVs in the healthy brain are indicative of rapid AV flux/turnover reflecting a highly active baseline autophagic machinery in neurons, rather than the low levels of basal autophagy that were earlier proposed.¹³ The most well-accepted role of basal autophagy is considered to be maintenance of cellular homeostasis, mainly by operating an efficient system for long-lived protein/organelle turnover. The most direct evidence in this regard has come from studies of neural-cell-specific *Atg5*^{−/−} and *Atg7*^{−/−} mice, which demonstrate a considerable loss of neurons (in a cell autonomous fashion), as well as an accumulation of ubiquitin-positive aggregates in the brain despite the apparently normal function of proteasomes.^{14,15} Given the importance of constitutive autophagy in cellular housekeeping and protein quality control, several studies have explored the link between aberrant autophagy and the onset of neurodegenerative disorders (see below).

Stress-Induced Autophagy

One of the classic roles for autophagy has been the cellular response to limited trophic factor support. Studies in early neonatal mice have firmly established the indispensability of autophagy in the supply of nutrients for survival,¹⁶ primarily through the generation of amino acids and/or the subsequent utilization of the latter to generate ATP. This has been further validated by the observation that a pharmacological agent, methyl pyruvate (trichloroacetic acid substrate), by virtue of its ability to maintain ATP production, can reverse the effect of growth factor deprivation in autophagy-deficient neurons that would otherwise die.¹⁷ Interestingly, analysis of GFP-LC3 transgenic mice has revealed that in response to starvation, while noticeable levels of GFP-LC3 AV-associated puncta are seen in liver, heart, and kidneys, very few puncta are seen in the brain, thereby leading to speculation that nutrient-stress induced autophagy may not be very pronounced in the central nervous system.¹⁸ However, unlike basal autophagy, this may indeed reflect low levels of autophagy induction, given the fact that the central nervous system is well-protected against short-term systemic starvation. Moreover, attempts to model starvation-induced autophagy in cultured primary neurons by classical methods have not been very successful, given the fact that withdrawal of serum and nutrients typically leads to rapid neuron death owing to their high energy demands. However, recently Young et al¹⁹ have effectively validated an *in vitro* model for starvation-induced autophagy in primary neurons that involves the selective withdrawal of nutrients. In this system, autophagy induction is triggered by the absence of insulin and proceeds via a mTOR-dependent mechanism. It remains to be determined if similar effects occur *in vivo*.

Oxidative stress-induced autophagy has been implicated in several neuropathological conditions. eg, following cerebral ischemia–hypoxia or traumatic brain injury, in which significant autophagy is seen in neurites and is accompanied by rapid and massive neurite degeneration.^{20,21} However, the presence of autophagosomes in degenerating neurons is not sufficient to establish a pathological role for autophagy. Typically, in response to oxidative stresses, damaged mitochondria release cytochrome c and other cell death modulators, triggering a cascade of events culminating in apoptotic death. However, selective autophagic clearance of damaged mitochondria can promote survival, thereby giving rise to a possible mechanism by which autophagy can protect against stress-induced death. Moreover, autophagy may prevent necrosis by catabolic energy generation.

On the other hand, a high level of “autophagic stress” may lead to massive lysosomal activation resulting in neuronal death.²⁰ In fact, a recent study has demonstrated that mice deficient in *Atg7* show considerable protection from both hypoxia/ischemia-induced caspase-3 activation and neuron death.²² Thus, the precise role of autophagy induction may depend on the death stimulus and cross talk between different death pathways. The resultant phenotype may be the cumulative effect of the events triggered by this cross talk.

Development-Associated Autophagy

Sculpting of the nervous system involves the loss of a large number of neurons, which has been considered to be tightly regulated by Type I programmed cell death (PCD) and characterized by apoptotic features such as chromatin condensation, membrane blebbing, and fragmentation into apoptotic bodies.²³ However, cells undergoing PCD may display morphological features distinct from apoptosis, and Type II (autophagic) PCD is one of several alternative types of PCD.

Type II PCD is defined by the prominent appearance of AVs in a dying cell. While the precise role of autophagy in neurodevelopment has not been completely elucidated, recently novel roles have emerged for autophagy in nervous system development. Beclin 1 has been found to be required for retinoic acid-induced differentiation of neuroblastoma N2a cells.²⁴ Moreover, in concert with studies on embryonic development, experiments on the retinal neuroepithelium have revealed that decreased ATP levels associated with deficient autophagy negatively affect degradation and removal of apoptotic cells. These cells fail to present ATP-dependent engulfment signals, such as the "eat-me" signal phosphatidyl serine, on the outer leaflet, and secrete low levels of the "come-get-me" signal lysophosphatidylcholine.^{25,26} Restoration of ATP levels by use of methyl pyruvate increased clearance of apoptotic cells and restored normal retinal development. This is in stark contrast to the decrease in ATP levels observed in response to trophic factor removal in autophagy-deficient cells, where the use of methyl pyruvate enhanced cell survival.¹⁷ However, the importance of autophagy in the ontogenesis of the central nervous system remains unclear because while *Ambra 1*^{gt} embryos display neural tube defects detected as midbrain/hindbrain exencephaly and/or spina bifida accompanied by excessive apoptosis,²⁷ transgenic mice deficient in *Atg5* or *Atg7* specifically in the central nervous system demonstrate no developmental abnormalities.^{14,15}

Aging

It is widely believed that while aging is a multifactorial process, accumulation of reactive oxygen species and the resultant oxidative damage occupies a central role in the age-related decline in cellular function. As mentioned previously, the long-lived, post-mitotic neuron is more susceptible to such oxidative damage. Given the role of autophagy in clearing damaged organelles and oxidized substrates, there have been several studies exploring the link between autophagy and aging. Experiments in a *Drosophila* model have demonstrated that levels of autophagy genes are significantly decreased in the neural tissues of older flies.²⁸ The resultant decrease in basal autophagy is accompanied by a concomitant increase in accumulation of insoluble ubiquitinated proteins. Enhanced *Atg8* expression in older fly brains extends the average adult lifespan by 56% and promotes resistance to oxidative stress and the accumulation of ubiquitinated and oxidized proteins. Secondly, the TOR pathway has been implicated in aging. In fact, low doses of

rapamycin have been shown to extend the chronological life-span of autophagy-competent yeast by up-regulation of autophagy.²⁹ This increase in longevity was not seen in autophagy-deficient yeast, thereby implying that rapamycin-mediated effects are dependent on *Atg* genes.

Neurodegenerative Disorders

A number of neurodegenerative disorders are characterized by accumulation of aggregate-prone proteins and are therefore collectively known as proteinopathies.³⁰ Several reports have clearly demonstrated that a deficiency in autophagy induction can lead to aberrant accumulation of neuronal proteins with subsequent onset of neurodegenerative disorders.³¹ On the other hand, it is now becoming increasingly clear that along with defective autophagy induction, impairment of autophagy completion can also lead to onset of neurodegenerative disorders. Defects may exist in the endocytic transport machinery or as a result of lysosomal dysfunction. We and others have shown that targeted disruption of cathepsin D (a major lysosomal protease) in mice results in aberrant accumulation of autophagosomes. This accumulation is accompanied by extensive neuropathology, including biochemical and morphological evidence of apoptosis—a phenotype closely resembling that seen in neuronal ceroid lipofuscinoses/Batten disease, which is a group of autosomal recessive diseases that together represent the most common form of pediatric neurodegenerative disease.^{32,33} Moreover, mutations in the autophagy-lysosomal pathway transport machinery have been shown to be associated with frontotemporal dementia and amyotrophic lateral sclerosis.³⁴ These and other studies have resulted in the identification of autophagy as a prospective therapeutic target for neurodegenerative disorders.

Enhancing autophagy as a therapeutic strategy is considered a better alternative than up-regulating proteasome activity since the latter degrades many key short-lived intracellular regulators like p53, whose altered degradation rates could have deleterious consequences.³⁰ In contrast, autophagy targets long-lived proteins whose steady state levels would not be considerably affected. Moreover, in the absence of steric considerations, autophagy would be predicted to be more effective in clearing large protein aggregates and oligomers. Proof of concept studies in cell models have aptly demonstrated that mutant huntingtin fragments, expanded polyalanines, and mutant forms of α -synuclein are autophagy substrates.³⁵ Clearance of these proteins is delayed by autophagy inhibitors and enhanced by rapamycin, which induces autophagy by inhibiting TOR. In fact, rapamycin and other inhibitors of TOR have been shown to confer protection in fly and mouse models of Huntington's disease and also in fly models of tauopathy (aberrant accumulation of tau protein eg, Alzheimer's disease). At least part of this protection is suggested to be through induction of autophagy.³⁶

Given the wide range of pathways that mTOR can regulate (including the control of protein synthesis), it is

unclear whether the protective effects of rapamycin can be solely attributed to autophagy. In fact, a recent study has demonstrated that rapamycin decreases expanded huntingtin and its inclusion bodies to a similar degree in both *Atg5*^{+/+} and *Atg5*^{-/-} mouse embryonic fibroblasts.³⁷ Moreover, it was observed that rapamycin actually reduced the amount of soluble protein input suggesting an effect on global protein synthesis, which was supported by the demonstration that cycloheximide (an inhibitor of protein synthesis) mimicked the protective effects of rapamycin. This raises the possibility that enhancers of autophagy working via mTOR may actually be exerting their effects through nonautophagy-related functions.

Recently an exciting theme has emerged with the identification of novel mTOR-independent pathways regulating autophagy, as well as drugs enhancing autophagy through these pathways eg, lithium. In fact, it has been shown that using modulators of TOR-dependent and -independent pathways in combination has additive effects on the autophagic clearance of mutant aggregates *in vivo*.³⁸ However, although typically up-regulation of autophagy is considered beneficial, a recent study using a cellular model of frontotemporal dementia has found that up-regulation of autophagy initiation only led to a greater accumulation of autophagosomes, presumably due to defective AV clearance, thereby increasing stress and subsequent neuronal loss.³⁹

Cancer

Although the precise role of autophagy in brain tumor initiation and therapy is ill-defined at present, it is becoming increasingly clear that whether autophagy inhibition promotes tumor growth or regression is a function of several variables, including the cell type, genotypic profile of the neoplastic cell, and the stage of oncogenesis being targeted. Stage-specific roles of autophagy have been proposed.⁴⁰ According to this model, autophagy can act as a suppressor of oncogenesis in precancerous stages by decreasing the overall genetic error load on the cell. This is achieved by the homeostatic function of autophagy whereby damaged mitochondria can be eliminated, thus reducing exposure of cellular DNA to genotoxic/mutagenic influences such as free radicals, and possibly limiting cellular transformation.

The identification of *beclin 1* as a tumor suppressor adds further support to this idea.⁴¹ In fact, cytoplasmic levels of beclin1 protein have been found to be decreased in high-grade versus low-grade glial neoplasms.⁴² Moreover, a positive correlation has been reported between beclin levels and apoptosis, while a negative correlation has been seen for proliferation rates. However, none of the *Atg* genes have been shown to have a similar role, and so it is still not certain if the tumor suppressive role of beclin-1 is indeed an autophagy-related effect. It is suggested that autophagy may effect turnover of proliferative/antiproliferative molecules and may serve to balance the levels of these

factors and hence keep a check on aberrant growth through autophagy-unrelated cross talk of the autophagy genes (*beclin 1* in particular) with the phosphoinositide 3-kinase pathway.⁴³

Therapeutic strategies targeting the different stages of the autophagy-lysosomal pathway are being tested. In fact, careful combination of different autophagy modulators and/or their addition to chemotherapeutic regimens may be an efficient means to treat drug-resistant tumors. This postulate has been successfully tested in several *in vitro* and *in vivo* models. A class of compounds that has shown great promise in this regard is lysosomotropic agents, which block autophagy completion. As the name indicates, these compounds accumulate in lysosomes, alter the activity of lysosomal enzymes, and block the degradation of AVs leading to their aberrant accumulation, which in turn has been shown by our lab and others to trigger death via caspase-dependent and -independent mechanisms.⁴⁴

Recently, we demonstrated that chloroquine, a prototypical lysosomotropic compound, can trigger AV accumulation and caspase-independent death in glioma cells.⁴⁵ Moreover, chloroquine was found to induce death in these cells regardless of their p53 status, which makes it an attractive therapeutic option given the fact that p53 is mutated in a significant percentage of gliomas and other tumors. In a relatively small human trial, chloroquine, when added to the standard treatment regimen of glioma patients, was found to increase their median survival time.⁴⁶

Lysosomotropic agents are also being effectively used in combination with autophagy-inducing compounds since coupling the latter with agents blocking autophagy at a late stage has been consistently reported to accelerate cell death in tumor cells. This therapeutic strategy may circumvent resistance to tyrosine kinase inhibitors such as imatinib, which have been found to induce autophagy in several cell types including glioma cells; imatinib-induced cytotoxicity was found to be enhanced by using bafilomycin A1 (blocks autophagy at a late stage).^{47,48} Similarly, knockdown of Akt, a survival kinase, has been shown to induce autophagy, which when blocked using chloroquine accelerates cell death in tumor cells.⁴⁹ Autophagy induction by Akt inhibition has also been shown to radiosensitize glioma cells.⁵⁰

In addition to its effect on modulating cell death, autophagy has also been shown to control the characteristics of dying cells. In a recent study on glioma cells, it was reported that dying cells in which autophagy had been induced selectively released the immune modulator protein high-mobility group B1 without causing classical necrosis, thereby giving rise to the possibility that modulating autophagy might also alter the immunogenicity of tumor cells.⁵¹

Conclusions

Recent studies have improved our understanding of the autophagic machinery, and identified diverse roles for

this physiological process in the nervous system. While this field is rapidly expanding, a word of caution needs to be sounded in the wake of the possibility that while the appearance of autophagosomes has been used to implicate autophagy in various physiological and pathological conditions, it may just be an “innocent bystander.” One important point that has emerged quite clearly is that the effective therapeutic targeting of autophagy requires a more detailed understanding of the stimulus-specific effects of autophagy, and the cross talk between pro-death and pro-survival pathways.

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