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Evaluation of Autophagy Using Mouse Models of Brain Injury

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SUMMARY

Autophagy is a homeostatic, carefully regulated, and dynamic process for intracellular recycling of bulk proteins, aging organelles, and lipids. Autophagy occurs in all tissues and cell types, including the brain and neurons. Alteration in the dynamics of autophagy has been observed in many diseases of the central nervous system. Disruption of autophagy for an extended period of time results in accumulation of unwanted proteins and neurodegeneration. However, the role of enhanced autophagy after acute brain injury remains undefined. Established mouse models of brain injury will be valuable in clarifying the role of autophagy after brain injury, and are the topic of discussion in this review.

Keywords

Autophagosome; Controlled cortical impact; Hypoxia-ischemia; Mitophagy; Traumatic brain injury

1. Introduction

Autophagy is a homeostatic, carefully regulated, and dynamic process for intracellular recycling of bulk proteins and aging organelles [1,2], and very recently discovered, lipids [3]. Alteration in the dynamics of autophagy has been observed in many diseases of the central nervous system (CNS). Increased autophagy has now been reported in experimental models of traumatic brain injury, stroke, and excitotoxicity [4–7], and in patients with Alzheimer's Disease and critical illness [8,9]. Mice with genetic disruption of autophagy develop accumulation of unwanted proteins and neurodegeneration, mimicking human conditions like Huntington's, Parkinson's, and Alzheimer's disease [10]. However, while lifetime disruption of autophagy is clearly deleterious, it remains unclear whether increased autophagy after critical injury or extended periods of cell stress is beneficial—or actually detrimental. Several

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studies using transgenic mice and/or established mouse models of brain injury have shed some light onto the role of autophagy after brain injury. However, the role of autophagy, particularly in the brain, remains controversial and is the topic of this review.

2. Autophagy

Autophagy occurs in all tissues and cell types, including the brain and neurons. Autophagy is an important cellular response to environmental stress. The classic stimulus for autophagy is starvation. Starvation-induced autophagy is initiated by extended nutrient deprivation, the hormone glucagon, and the second-messenger cAMP [11,12]. Autophagy is a highly regulated process requiring the ubiquitin E1-like enzyme Atg7 (autophagy-associated proteins abbreviated "Atg"), phosphorylation of pre-autophagosomal membranes, formation of Atg12-Atg5 complexes, and processing of Atg8/microtubule-associated protein light chain-3 (*LC3*, is the mammalian homologue of the yeast *Atg8*), and covalent attachment of phosphatidyl-ethanolamine to LC3-I resulting in LC3-II (Figure 1). LC3-II is a relatively specific biochemical footprint of autophagy, referred to as an "LC3 shift" or "LC3 lipidation" [13]. These processes culminate in the formation of autophagosomes containing cytoplasmic material and/or organelles such as mitochondria. Autophagosomes fuse with lysosomes, facilitating enzymatic disassembly of large proteins and organelles leading to recycling of amino acids and other nutrients [14]. Starvation-induced autophagy is regulated by class III phosphatidylinositol 3-kinase (PI3-K) and the Bcl-2 interacting partner, Beclin-1 [15]. In contrast, regulation of autophagy may differ in non-starvation conditions. For example, the mitogen activated protein kinase/extracellular signal regulated protein kinases (MEK), rather than PI3-K, appear to regulate autophagy triggered by oxidative stress as the (MEK) inhibitor 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126), but not the class III PI-3K inhibitor 3-methyladenine, reduces autophagy induced by 1-methyl-4-phenylpyridinium [16]. Importantly, oxygen radicals are also essential for autophagy to proceed normally [17].

3. Mouse Model to Monitor Autophagy

As briefly noted above, LC3-II is a reliable biomarker of autophagy. Taking advantage of this, Mizushima et al. developed a transgenic mouse where LC3 is tagged with green fluorescent protein (GFP) [18]. GFP-LC3 is concentrated in autophagosomes, and thus formation of GFP-enriched vesicles can be used to monitor autophagy [1]. Hemizygous expression of *GFP-LC3* does not itself affect autophagy rates, and GFP-LC3 localizes to autophagosome membranes upon stimulation of autophagy similar to endogenous LC3 [19]. GFP fluorescent macroscopy can be used to identify transgenic embryos (Figure 2A). *GFP-LC3*$^{+/-}$ transgenic mice can be used to monitor autophagy both in vivo and in vitro. Mizushima et al. evaluated *GFP-LC3*$^{+/-}$ mice and found that the response to nutrient deprivation was tissue-specific [18]. In some tissues, such as the brain, ovaries, and testes, an increase in autophagy after 48 hours of starvation was not detectable. In others, such as the kidney, pancreas, and thymus, baseline autophagy was observed in some cell types. In most tissues, particularly the liver and muscle, 48 hours of nutrient deprivation produced a reliable and robust increase in autophagosome formation.

The value of these mice in the dynamic assessment of autophagy in neurons, was first demonstrated in an in vitro model of starvation. Depriving neurons of D-glucose, sodium pyruvate, L-glutamate, L-glutamine, L-aspartate, and supplements containing protein and lipid, leads to formation of GFP-LC3 enriched vacuoles (consistent with autophagosomes) within an hour (Figure 2) [20]. Cultures from these mice can be used to track fusion of autophagosomes with lysosomes [20] and mitochondria (Figure 2C). In contrast to fibroblasts, inhibition of autophagy using 3-methyladenine—a classic but non-specific inhibitor of autophagy, or siRNA

targeting Atg7, increased neuronal survival, suggesting that in non-dividing cells such as neurons, increased autophagy is deleterious. Another interesting finding in this study was that during starvation, induction of autophagy was sex-dependent [20]. Neurons from male mice show a robust increase in autophagosomes versus their female counterparts during starvation. Both resistance to starvation and decreased autophagy in neurons was associated with the capacity of neurons from females to mobilize lipid sources and form lipid droplets. Very recently, a critical link between LC3 lipidation and autophagy of lipid droplets was reported, a process coined “lipophagy” [3].

Because 48 hours of food-deprivation does not produce detectable autophagy in brains from mice [18], the in vivo relevance of this in vitro starvation model, can be questioned. However, studies in humans with prolonged periods of profound starvation—those undergoing voluntary hunger strikes and patients with anorexia nervosa, show radiographic evidence of brain tissue loss, consistent with some degree of autophagy and/or lipophagy in brain [21–23]. Since it would be difficult to justify subjecting animals to extended periods of starvation, this question may never be answered. Nevertheless, what is clear is that neurons retain the capacity to undergo autophagy, the role of autophagy in response to stress may be different in neurons compared to other tissues—particularly those that also serve as nutrient depots such as adipose, liver, and skeletal muscle, and that the autophagic response to stress is sex-dependent.

4. Mouse Models of Disrupted Autophagy

4.1. Atg7 deficient transgenic mice

In 2005, Komatsu et al. reported generation of conditional knockout mice deficient in *Atg7* [24]. They found that mice with completely lacking *Atg7* die within 1 day after birth. The following year, this group reported generation of mice with tissue-specific *Atg7* knockdown in the CNS [10]. These mice had growth retardation, motor and behavioral deficits, and extensive neuronal loss, dying within 28 weeks of birth. Additionally, age-dependent increases in ubiquitin-containing inclusion bodies were present in neurons reflecting disrupted autophagy, and correlating with neurodegeneration and neurological dysfunction.

Koike et al. evaluated the influence of CNS *Atg7* deficiency in a mouse model of neonatal hypoxia-ischemia [25]. This adaptation of the Levine preparation [26] is often referred to as the Rice-Vannucci model when used in immature rodents [27], and involves unilateral carotid artery ligation followed by a period of hypoxia. This insult can be done in rodents of very young age, including mice, and produces a unilateral brain infarction. Neonatal *Atg7* deficient mice had less hippocampal neuronal death and autophagy compared with their wild-type counterparts, implying that autophagy contributes to neurodegeneration after acute cerebral ischemia. Importantly, the protective effect of *Atg7* deletion is not seen in older mice, suggesting that any advantage *Atg7* mice may have in response to ischemia is counterbalanced by accumulation of proteins with increasing age related to disrupted autophagy.

4.2. Atg5 deficient transgenic mice

In 2004, Kuma et al. reported generation of homozygous mice deficient in $Atg5^{-/-}$ [28]. These mice appeared normal at birth, yet most died within 1 day of life. Atg5 and Atg12-Atg5 conjugates were undetectable in these mice. LC3-II was significantly reduced or absent, and LC3-I was increased. In neural-cell-specific $Atg5^{-/-}$ mice, development of progressive motor and behavioral deficits and degenerative changes in neurons is observed, again illustrating the necessity of autophagy in cellular homeostasis.

5. Mouse Models of Enhanced Autophagy

Both transgenic and non-transgenic mouse models have been used to provide valuable insight into the pathobiology and pathophysiology of acute brain injury [29]. Recently, contemporary mouse models of traumatic and ischemic brain injury have shown increased autophagy, and have begun to shed some light—or perhaps a cloud of controversy, as to the role of autophagy in the mature and developing mammalian brain. Specifically, whether autophagy is beneficial and/or detrimental, or merely an epiphenomenon after acute brain injury.

5.1. Traumatic brain injury

The first report that traumatic brain injury may stimulate autophagy was by Diskin et al. in 2005 [30]. Using a mouse model of closed head injury produced by dropping a weight onto the intact skull, they assessed changes in Beclin-1, a bcl-2 interacting partner that regulates starvation-induced autophagy. They found that while Beclin-1 levels are relatively low in brain from uninjured animals, Beclin-1 increased in neurons and activated astrocytes at the site of injury [30]. Thus, providing indirect evidence that autophagy is increased after traumatic brain injury. Investigators from this laboratory later evaluated the effects of treatment with rapamycin in this closed head injury model [31]. Rapamycin triggers autophagy via inhibition of mammalian target of rapamycin (mTOR) and consequential disinhibition and activation of PI3-K (Figure 1A). Rapamycin was developed as an anti-fungal agent, and also has anti-inflammatory effects and the ability to cause cell cycle arrest and inhibit cell proliferation [32]. Erlich et al. [31] found that intraperitoneal injection of rapamycin administered 4 hours after injury resulted in improved neurobehavioral function as determined by a neurological severity score and increased neuronal survival in the injured region compared to vehicle treated mice. Additionally, western blot showed increased Beclin-1 at 5 hours in with rapamycin treatment, consistent with an augmented autophagic response. Collectively, these data support rapamycin-enhanced autophagy producing beneficial neurological effects after traumatic brain injury. Although, non-autophagic salutatory effects of rapamycin cannot be discounted.

Ultrastructural identification of autophagosomes remains the “gold standard” method for detection of autophagy [1]. As such, the first direct evidence that autophagy is increased after traumatic brain injury was reported by Lai et al. using a controlled cortical impact model of traumatic brain injury [4]. Controlled cortical impact is a contemporary model of traumatic brain injury that produces a contusion with cortical necrosis, delayed neuronal death in brain regions such as the hippocampus and thalamus, and sustained motor and cognitive deficits [33]. An increase in autophagosomes—vacuoles, multilamellar bodies, and secondary lysosomes, was observed from 2–48 hours after traumatic brain injury. Biochemical evidence of autophagy, increased LC3-II levels in injured brain was also reported. As oxidative stress can induce autophagy [17] and oxygen radicals are essential for autophagy to proceed normally [16], the effect of the cysteine-donor antioxidant γ-glutamylcysteinyl ethyl ester (GCEE) was evaluated. Systemic administration of GCEE after injury resulted in reduced autophagy as determined by LC3-II formation, increased antioxidant reserves, improved cognitive performance, and reduced histological damage compared with vehicle treatment. The same caveat mentioned above regarding non-autophagy related effects of rapamycin also applies to GCEE. However, there does appear to be a link between autophagy and oxidative stress after traumatic brain injury. Oxidative stress is a prominent feature of traumatic brain injury in mice [34] and humans [35].

5.2. Hypoxia-ischemia

Similar to traumatic brain injury, autophagy was also reported to be upregulated after hypoxic-ischemic injury in mice in 2005. Zhu et al. examined the effects of age and hypoxia-ischemia induced by unilateral carotid artery occlusion and hypoxemia (Rice-Vannucci model) on LC3-

II levels as a biomarker of autophagy [6]. They detected levels of LC3-I and LC3-II in control brains, and found that younger (post-natal day 5; PND 5) animals had higher levels of both LC3-I and LC3-II compared with older animals (PND 60). Thus, similar to apoptosis, autophagy appears to be age-dependent and more prominent during early stages of development. After unilateral hypoxia-ischemia, relative LC3-II abundance was increased in the ipsilateral hemisphere, with the greatest increase in adult brains [6]. The following year, this group also reported that this increase in LC3-II was not sex-dependent in this model of neonatal hypoxia-ischemia [36].

Autophagic neurodegeneration is also seen after cerebral ischemia in adult mice. After transient middle cerebral artery occlusion, Degterev et al. showed an increase in autophagy that was not affected by the novel "necroptosis" inhibitor Necrostatin-1 [5]. Adhami et al. reported the effect of unilateral carotid occlusion with hypoxemia in adult mice [37]. They noted the presence of vacuole-associated damage via electron microscopy consistent with autophagy in the ischemic brain. Using GFP-LC3 transgenic mice, they also showed increased punctate GFP immunolabeling at 6 and 18 hours after hypoxia-ischemia. An increase in LC3-II was not detected after hypoxia-ischemia; however, a transient reduction in LC3-I and depletion of cytosolic LC3 was observed.

6. Reconciliation of Current Studies

To date, mouse models have been valuable in establishing that autophagy is a vital homeostatic process, and that disrupted autophagy in normal tissues—including in the brain, is detrimental [10,28]. Contemporary mouse models have also established that the autophagic response can be triggered in brain by trauma [4], ischemia [5,6], and excitotoxicity [7]. However, the role of autophagy—beneficial, detrimental, or an epiphenomenon, after acute brain injury remains uncertain and controversial [38,39].

6.1. If not enough autophagy is bad, is more autophagy better?

Undoubtedly autophagy is essential for the clearance and recycling of accumulated proteins and aging or dysfunctional organelles. Disruption of autophagy, especially for the lifetime of an animal leads to accumulation of proteins. If accumulation of the particular protein or the protein itself happens to be toxic, then functional autophagy is of greater importance to cellular homeostasis. Examples of toxic protein accumulation associated with CNS pathology include mutated Tau, β-amyloid, α-synuclein, and Huntington protein [40]. As mentioned above, mice with disrupted autophagy due to deletion of *Atg5* or *Atg7* demonstrate neuropathology with accumulated protein aggregates and phenotypic changes mimicking human neurodegenerative disease [10,28]. Extrapolating to diseases where increased autophagy is evident, perhaps this increase is designed to clear protein aggregates? For example, β-amyloid—sufficient to induce neuronal apoptosis [41], is known to accumulate after traumatic brain injury [42].

On the other hand, since baseline autophagy in neurons is low (indeed rarely detected) and protein synthesis in general is inhibited after acute brain injury, increased autophagy may not be necessary for clearance of protein. In non-human primates after global brain ischemia, protein synthesis in all brain regions is inhibited within hours after ischemia [43]. In some regions, such as the cerebellum and cortex, protein synthesis recovered within 24 hours. In others, such as the hippocampus, thalamus, and arterial border zones of the cortex, protein synthesis was inhibited by as much as fifty percent compared with normal controls. Additionally, chaperone proteins are not subject to this general reduction in protein synthesis after ischemia [44]. Signals for autophagic degradation of proteins also include sequence specific regions (KFERQ) that interact with chaperone proteins [45]. Chaperone-assisted degradation of accumulated proteins, versus autophagosome-lysosome mediated degradation of organelles and bulk proteins, may play different roles after acute brain injury.

Another unanswered question is whether increased autophagy participates in the clearance of reversibly or irreversibly damaged organelles, or whether its consumption of organelles such as mitochondria in cells with low baseline autophagy is indiscriminate? Mitochondrial fission, which likely occurs before engulfment into autophagosomes (Figure 3), is not necessarily irreversible after injury [46]. Enhanced autophagy may contribute to consumption of potentially viable or uninjured organelles such as mitochondria after fission, and therefore, may exacerbate energy failure and worsen injury. Mitochondrial dysfunction [47,48] and energy failure [49] are prominent features of both traumatic and ischemic brain injury.

Addressing this question directly has been hampered by the fact that studies in autophagy-related transgenic mice are confounded by progressive neurodegeneration as the mouse ages, and the lack of specific pharmacological agents targeting autophagy. In the study by Koike et al. using *Atg7* deficient mice, cerebral ischemia was imposed on PND 7, and brains were evaluated 3 days later [25], long before neuropathology is detectable in these mice [10]. The studies by Erlich et al. [31] and Lai et al. [4] testing pharmacological therapies that increase and inhibit autophagy, respectively, in mouse models of traumatic brain injury were conflicting. Rapamycin, which induces autophagy via inhibition of mTOR improved neurological outcome [31]; whereas the antioxidant GCEE reduced autophagy but also improved neurological outcome [4]. As noted above, both of these studies tested drugs with multiple effects in addition to those related specifically to autophagy. A more recent study in a rat model of transient focal cerebral ischemia in PND 12 rats [50], tested what was regarded as a more selective inhibitor of autophagy, the class III PI3-K inhibitor 3-methyladenine [12]. Post-treatment with 3-methyladenine administered intracerebroventricularly reduced infarction size compared with vehicle treatment and caspase inhibitors, which had no effect [50]. Similar to rapamycin and GCEE, however, 3-methyladenine also has other effects including inhibition of non-class III PI3-kinases and promotion of glycogen breakdown in hepatocytes [51]. Taken together, these pharmacological studies tend to cloud, rather than clarify, the role of autophagy after acute brain injury.

6.2. Is the role for autophagy cell-type dependent?

Another important question is whether the role of autophagy in neurons and/or brain is different from other cell-types and tissues? Autophagy at baseline and during starvation is clearly tissue-dependent [18]. It is interesting to note that tissues that also serve as nutrient depots, protein in muscle and glycogen in liver, tend to have robust autophagic responses that are probably beneficial to the organism as a whole. While curiously evidence for GFP-enriched vesicles were not routinely observed in fat [18], recent evidence suggests that regulation of lipid during starvation may be via an autophagic process [3]. In contrast, typically non-dividing cells such as neurons, spermatocytes, and oocytes have minimally detectable autophagy at baseline, and do not have an autophagic response to 48 hours of starvation [18]. Inhibition of autophagy during nutrient deprivation reduces cell death in neurons, but increases cell death in fibroblasts [20], suggesting that not only is the autophagic response tissue-dependent, but the role of autophagy is as well.

7. Conclusions

While mouse models have helped establish the presence of autophagy in brain after acute injury, they have not yet helped clarify the role of autophagy. However, it seems clear that either too little, or too much, autophagy is detrimental to normal homeostasis and the response to injury, and that this is tissue- and cell-type dependent. In this regard, autophagy is quite like apoptosis. Indeed, many other similarities and cross-talk occur between autophagy and apoptosis [52]. Important unanswered questions remain, that will require development and

testing of specific interventions that either increase or inhibit autophagy in injured brain, before these questions can be answered.

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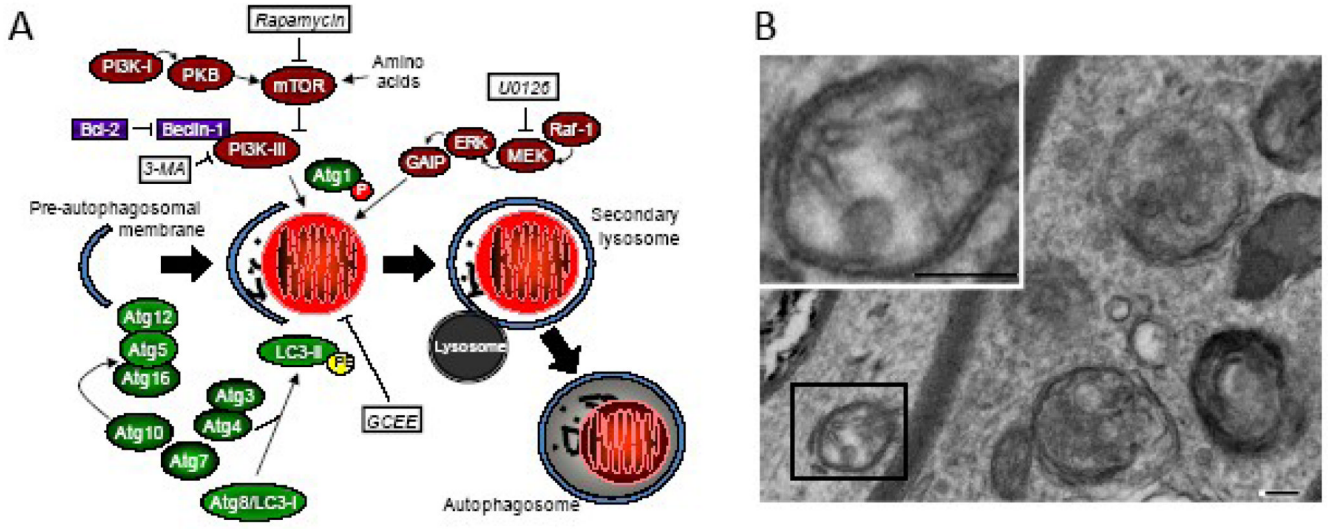

Fig. 1.

A. Simplified schematic of starvation- and non-starvation-induced autophagy. Putative effectors of autophagy are highlighted in white boxes. *B.* Electron micrograph showing autophagosomes (t he “gold standard” for detection of autophagy) in an axons after traumatic brain injury in mice (bar = 100 nm). ***Abbreviations:*** 3-MA, 3-methyladenine; Atg, autophagy complex component; ERK, extracellular signal regulated protein kinase; GAIP, Gα-interacting protein; GCEE, γ-glutamyl cysteine-ethyl ester; LC3, light chain 3; MEK, mitogen activated protein kinase/ERK; mTOR, mammalian target of rapamycin; P, phosphate; PE, phosphatidylethanolamine; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; U0126, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene.

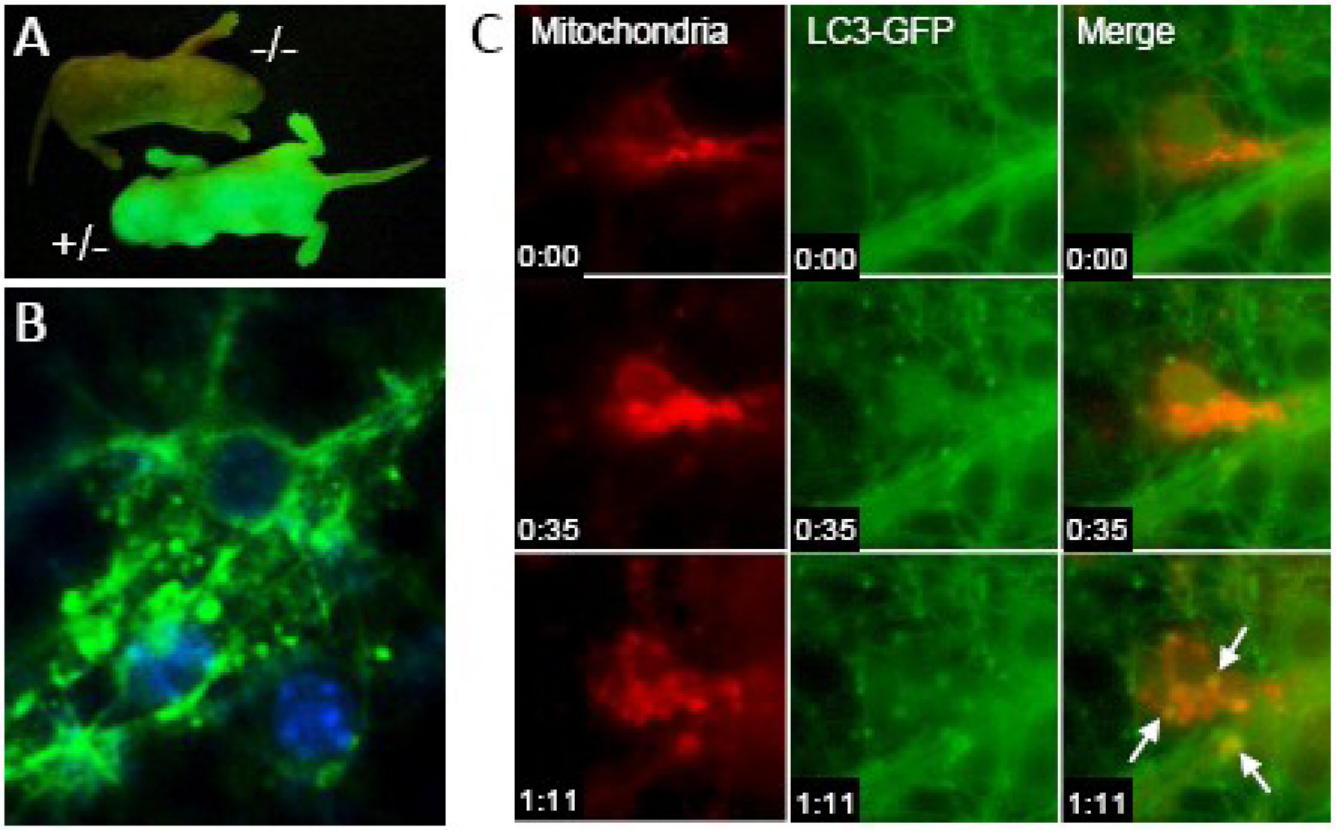

Fig. 2.

Dynamic tracking of autophagy in primary cortical neurons in vitro. *A*. Discrimination of *GFP-LC3*$^{+/-}$ mice using fluorescent macroscopy. *B*. Identification of GFP-LC3-enriched vesicles suggestive of autophagosomes in primary cortical neurons from *GFP-LC3*$^{+/-}$ mice after 24 hours of nutrient deprivation using fluorescent microscopy. *C*. Dynamic tracking of mitophagy in primary cortical neurons from *GFP-LC3*$^{+/-}$ mice using time-lapsed microscopy. Autophagosomes (green) fusing with mitochondria (red; MitoTracker Red, Invitrogen, Carlsbad, CA) are highlighted by arrows. Time stamp is hours:minutes.

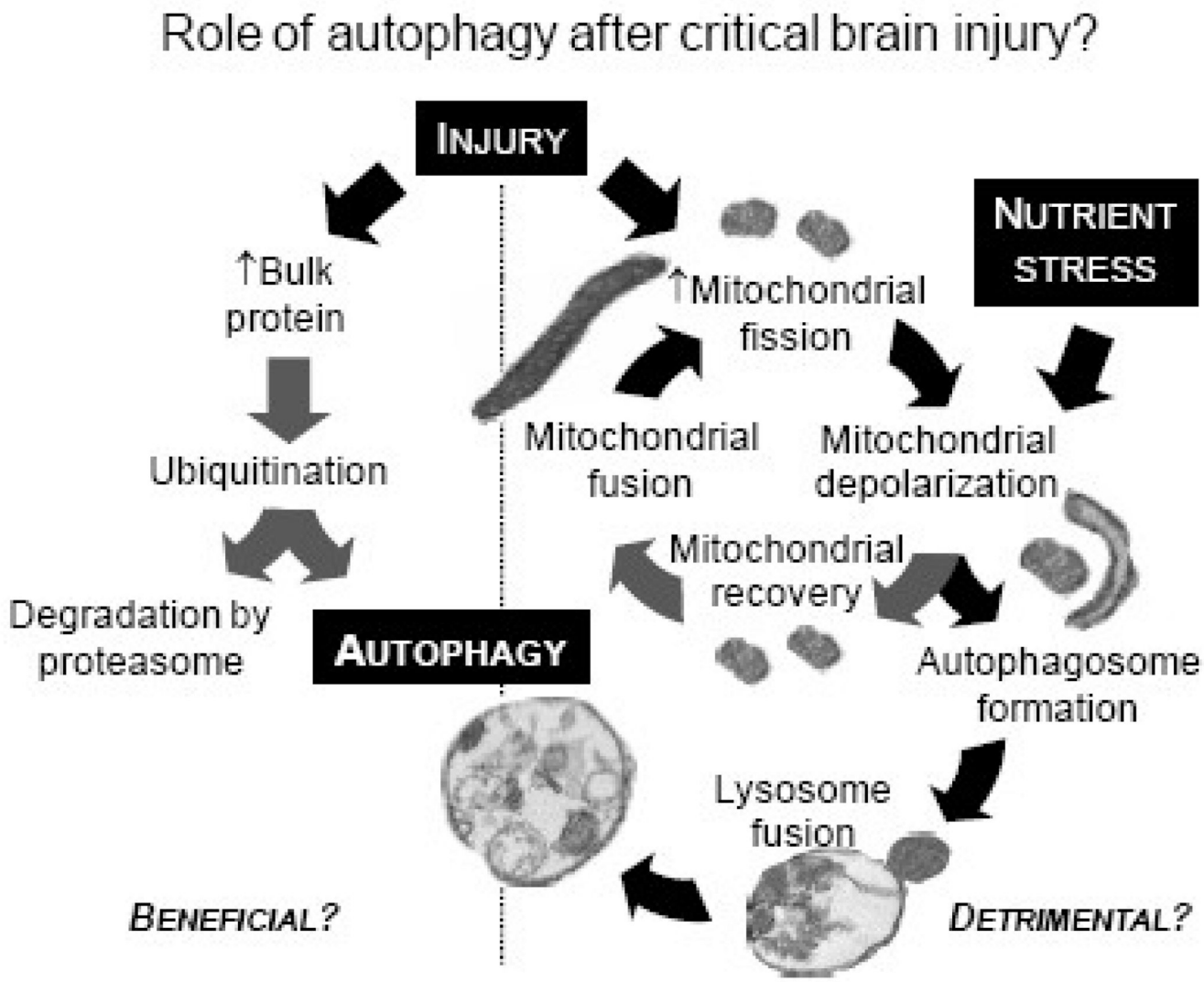

Fig. 3.
Hypothetical schematic illustrating potential dual-roles of autophagy after critical brain injury.