Published in final edited form as:

Neurobiol Dis. 2011 July ; 43(1): 52–59. doi:10.1016/j.nbd.2010.09.014.

Autophagy in Acute Brain Injury: Feast, Famine, or Folly?

Craig M. Smith^a, Yaming Chen^a, Mara L. Sullivan^b, Patrick M. Kochanek^{a,c}, and Robert S. B. Clark^{a,c,*}

^a Department of Critical Care Medicine, Safar Center for Resuscitation Research, Brain Trauma Research Center, and Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

^b Department of Cell Biology and Physiology, Safar Center for Resuscitation Research, Brain Trauma Research Center, and Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

^c Department of Pediatrics, Safar Center for Resuscitation Research, Brain Trauma Research Center, and Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

Abstract

In the central nervous system, increased autophagy has now been reported after traumatic brain and spinal cord injury, cerebral ischemia, intracerebral hemorrhage, and seizures. This increase in autophagy could be physiologic, converting damaged or dysfunctional proteins, lipids and/or organelles to their amino acid and fatty acid components for recycling. On the other hand, this increase in autophagy could be supraphysiologic, perhaps consuming and eliminating functional proteins, lipids and/or organelles as well. Whether an increase in autophagy is beneficial (feast) or detrimental (famine) in brain likely depends on both the burden of intracellular substrate targeted for autophagy and the capacity of the cell's autophagic machinery. Of course, increased autophagy observed after brain injury could also simply be an epiphenomenon (folly). These divergent possibilities have clear ramifications for designing therapeutic strategies targeting autophagy after acute brain injury, and are the subject of this review.

Keywords

Autophagosome; Autophagic stress; Hypoxia-ischemia; Lipophagy; Mitophagy; Traumatic brain injury

Introduction

To the organism as a whole, autophagy is a dynamic and carefully regulated process for intracellular maintenance of proteins, lipids, and aging organelles (Klionsky et al., 2008; Singh et al., 2009; Todde et al., 2009). During times of nutrient availability, autophagy is a homeostatic process, recycling dysfunctional or aging macromolecules and organelles back to their amino acid and fatty acid building blocks. During times of nutrient deprivation,

*Correspondence: Robert S. B. Clark, M.D., Safar Center for Resuscitation Research, 3434 Fifth Avenue, Pittsburgh, PA 15260, Telephone: 412-383-1900, Fax: 412-692-6076, clarkrs@ccm.upmc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

autophagy increases, going beyond physiologic recycling of aging macromolecules and organelles, perhaps consuming functioning proteins and organelles to provide additional fuel for the cell itself and potentially adjacent and distant ones as well. Thus preserving the cell (and potentially the whole organism) until famine has subsided and nutrients are replete.

In the mature mammalian brain, there is very little detectable autophagy (Mizushima et al., 2004); although this could represent highly efficient turnover of autophagosomes rather than low basal levels (Boland and Nixon, 2006). The mammalian brain is also felt to be protected from systemic nutrient deprivation, and autophagy is not observed in brains from mice deprived of food for 48 hours (Mizushima et al., 2004). However, it is possible to trigger autophagy in neurons during extreme nutrient deprivation, i.e. starvation (from *sterven*, *steorfan*, or *sterban* “to die”), in vitro (Du et al., 2009). Despite the controversy on whether nutrient deprivation can trigger autophagy in brain in vivo, there is consensus that other acute insults can rapidly increase autophagy in brain. Increased autophagy has now been reported in experimental models of traumatic brain injury (TBI), cerebral ischemia, and excitotoxicity (Degterev et al., 2005; Lai et al., 2008; Shacka et al., 2007; Zhu et al., 2005), and in patients with critical illness (Clark et al., 2008). In regard to autophagy after acute brain injury, two questions arise. First, what is the cellular autophagic burden, and second, what is autophagy’s role?

Impact of disrupted autophagy in brain

There is no question that disrupting the autophagic process in brain, particularly for the lifespan of the animal, is deleterious, resulting in the accumulation of dysfunctional or aging macromolecules and organelles (Fig. 1). Mice deficient in *Atg5* or *Atg7* die within 1 day after birth (Komatsu et al., 2005). Mice with central nervous system (CNS)-specific *Atg7* knockout have growth retardation, motor and behavioral deficits, and extensive neuronal loss, dying between 4 and 28 weeks of birth (Komatsu et al., 2006). In these mice, a duration-dependent increase in ubiquitin-containing inclusion bodies within neurons (reflecting disrupted autophagy) correlated with neurological dysfunction.

It is unclear, however, whether *acute* disruption of autophagy is detrimental or beneficial. It is likely that short durations of disrupted autophagy are well tolerated, but that longer durations are not. What this duration may be is entirely unknown, and likely depends upon the condition of the brain and co-morbidities. For example, if accumulation of the particular protein or the protein itself happens to be toxic, then functional autophagy is of greater importance and disruption even for a brief period of time may not be tolerated. These proteins may include mutated Tau, β - amyloid, α -synuclein, and Huntington protein (Goedert and Jakes, 2005). In the presence of these toxic proteins and/or their aggregates, increased autophagy may in fact be an endogenous protective mechanism attempting to clear these toxic substances. The presence of toxic proteins within cells would add to autophagic stress and increase the physiologic role of autophagy (Fig. 1). For example, β -amyloid accumulates after TBI (Ikonomic et al., 2004) and if not cleared is sufficient to induce neuronal death (Morishima et al., 2001). Indeed, previous TBI is a risk factor for Alzheimer’s disease (Jellinger, 2004) and β -amyloid plaques are one pathologic feature (Ikonomic et al., 2004).

Autophagy after TBI

What is the autophagic burden after TBI?

The first report of increased autophagy after TBI can be credited to Diskin, et al. (2005). They found that the bcl-2 interacting partner and autophagy regulating protein Beclin-1 is increased in neurons and astrocytes in mice after closed head injury (Diskin et al., 2005).

Compared with the uninjured hemisphere, an extraordinarily high number of cells had an increase in Beclin-1 immunoreactivity at the site of impact. These authors also reported that 17–37% of Beclin-1 immunoreactive cells in the injured hemisphere were also terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive; implying that a fair proportion of dying cells were undergoing some degree of autophagy.

The first ultrastructural identification of autophagosomes after TBI was reported by Lai, et al. (electronic publication September 5, 2007) using a controlled cortical impact model in mice. An increase in autophagosomal vacuoles, multilamellar bodies, and secondary lysosomes, was observed from 2–48 hours after TBI in the ipsilateral parietal cortex and dorsal hippocampus (Lai et al., 2008). While electron micrographic data are notoriously hard to quantify, particularly when it comes to double-membrane structures in the brain given the vast number of neurites and other cell processes (Chu et al., 2009); the relative amount of microtubule-associated protein-1 light chain 3-II (LC3-II) was increased by over 100% in injured versus uninjured brain (Lai et al., 2008). Formation and/or turnover of LC3-II from LC3-I is regarded as reliable biochemical evidence of autophagy (Kabeya et al., 2000), with the caveat that later-stage components of the autophagy pathway must be fully functioning. LC3-II accumulation may occur as a consequence of reduced degradation rather than increased autophagy, and it is undetermined whether this is the case after TBI.

Increased autophagy has also been reported in rat models of TBI. Using a fluid percussion injury model, Liu et al. (2008) showed that relative levels of LC3-II were increased by 100–200% in injured brain compared with sham rats (Liu et al., 2008). They also found that dying cells identified using propidium iodide (PI) staining and confocal microscopy generally did not co-label with antibodies directed against LC3, leading them to speculate that autophagy was protecting viable (LC3 positive/PI negative) cells. After controlled cortical impact injury in rats, relative levels of LC3-II are consistently increased by 100–200% between 2 and 48 hours (Sadasivan et al., 2008). LC3 immunoreactivity is also increased several-fold in rats after controlled cortical impact compared with sham injury, peaking at 8 days (Zhang et al., 2008).

Given developmental differences in autophagy in both normal and injured brain (Zhu et al., 2005), and sex-differences in the neuronal response to nutrient deprivation in vitro (Du et al., 2009), we characterized autophagy using a “pediatric” TBI model in both male and female rats. Moderate controlled cortical impact injury in postnatal day (PND) 17 rats was performed as described (Bayir et al., 2007) under an Institutional Animal Care and Use Committee approved protocol. PND 17 rats are used because they are developmentally similar in terms of cerebral metabolism, neurogenesis, and synaptogenesis to an approximately 1–4 year old child (Rice and Barone, 2000). Anesthetized PND 17 rats were subjected to controlled cortical impact (2.5 mm depth of penetration; 4 m/s velocity; 50 msec duration) to the exposed left parietal cortex. At 24 or 48 h, the rats were re-anesthetized and perfused, and brains were harvested for western blot analysis, immunohistochemistry, or electron microscopy. Western blot and immunohistochemistry were performed using an antibody that identifies both LC3-I and -II (MBL International, Woburn, MA, USA). Western blot showed that LC3-II is increased several fold at 24 and 48 h after TBI versus naïve controls, and that this increase is more prominent in male versus female rats (Fig. 2A). Immunohistochemistry showed that after TBI, LC3 labeling changed from a diffuse to a relatively more punctate pattern consistent with autophagosome formation in the ipsilateral cortex, CA3 region of the hippocampus, and thalamus (Fig. 2B), all regions vulnerable to neuronal death after controlled cortical impact injury (Clark et al., 1997). Furthermore, autophagosomal vacuoles, multilamellar bodies, and secondary lysosomes were seen in these regions by electron microscopy (Fig. 2C-G). These data suggest that traumainduced autophagy is not limited to the mature mammalian brain, and

that similar to nutrient deprivation studies in vitro (Du et al., 2009), there are sex-dependent differences in the autophagic response.

Taken together (and with the caveat that increased LC3-II may represent reduced turnover rather than increased autophagy in mind) there are multiple lines of evidence to suggest that there is a robust increase in autophagy in the injured rodent brain after trauma. There is also circumstantial evidence that autophagy is increased in humans after TBI as well (Clark et al., 2008). Thus the degree of autophagy triggered by TBI seems substantial and may represent a heavy cellular burden. Whether or not this increased autophagic response to the added cellular burden is beneficial in the long run—clearing damaged proteins and organelles, reducing mitochondrial energy consumption, recycling macromolecules; or detrimental—consuming functional macromolecules and organelles and exacerbating cellular stress, likely depends upon the capacity of the cell's autophagic machinery and ability to tolerate the added autophagic burden.

What is the role of autophagy after TBI?

Pinning down the role of autophagy after TBI has been hampered by the lack of specific pharmacological agents targeting autophagy, and the fact that transgenic mice with disrupted autophagy develop a neurodegenerative disease phenotype around 4 weeks of age (Komatsu et al., 2006). As such, the precise role of autophagy after TBI relies on extrapolation from studies using non-selective agents targeting mechanisms that include autophagy. As it turns out, these studies have yielded diametrically opposing viewpoints as to the role of autophagy after TBI.

Support for a *beneficial* role of autophagy after TBI is based on effects of treatment with rapamycin. Rapamycin induces autophagy via inhibition of the mammalian target of rapamycin (mTOR) and consequential disinhibition and activation of phosphoinositide 3-kinase (PI3-K). Rapamycin is a product of the bacterium *Streptomyces hygroscopicus* found on the island of Rapa Nui (Easter Island), is used clinically as an immunosuppressive drug, and also has antiinflammatory effects and the ability to cause cell cycle arrest and inhibit cell proliferation (Sehgal et al., 1975). A single intraperitoneal injection of rapamycin (0.5 or 1.0 mg/kg) administered 4 hours after controlled cortical impact injury in rats resulted in improved neurobehavioral function as determined by a neurological severity score compared with vehicle treatment (Erlich et al., 2007). Increased neuronal survival in injured brain was also reported with rapamycin treatment, with 677 neurons counted (identified using NeuN staining) versus 218 neurons counted in vehicle treated rats. However, it was not made clear in that study what the neuronal counts are in uninjured mice, so the survival benefit of rapamycin after TBI cannot be calculated. In addition, rapamycin treatment increased Beclin-1 at 5 hours detected via western blot, consistent with an augmented autophagic response, and reduced inflammation and gliosis versus vehicle treatment (Erlich et al., 2007). Collectively, these data support rapamycin- enhanced autophagy producing beneficial neurological effects after TBI; although nonautophagic or upstream salutatory effects of rapamycin cannot be discounted.

A potential beneficial role for autophagy is also implicated in studies where animals are fasted after TBI (Davis et al., 2008). Twenty four, but not 48, hours of fasting after controlled cortical impact in rats resulted in brain tissue sparing and improved performance in the Morriswater maze (a test of spatial memory). However, extrapolating these data to evidence in support of favorable effects of autophagy after TBI may be problematic, given that 48 hours of fasting is insufficient to induce autophagy in brain—albeit in mice versus rats (Mizushima et al., 2004). However, a recent and very elegant study by Alirezaei et al. (2010) has disputed these data. GFPLC3 transgenic mice (similar to those used by Mizushima et al., 2004) were fasted for 24 to 48 hours. A “dose-dependent” increase in

autophagosomes was detected in cerebellar Purkinje cells and cerebral cortical neurons using confocal and electron microscopy in fasting compared with fed mice (Alirezai et al., 2010). Caution is in order given the potential of selection bias with these methodologies and relatively small sample sizes ($n = 2$ or 3 mice/group). None-the-less, this report does challenge existing dogma.

Support for a *detrimental* role of autophagy after TBI is based on effects of treatment with the cysteine-donor antioxidant γ -glutamylcysteinyl ethyl ester (GCEE). Oxidative stress induces autophagy (Scherz-Shouval et al., 2007) and oxygen radicals are essential for autophagy to proceed normally (Zhu et al., 2007). A single intraperitoneal injection of GCEE (150 mg/kg) administered 10 min after controlled cortical impact injury in mice resulted in reduced autophagy as determined by LC3-II formation, increased antioxidant reserves, and improved Morris-water maze performance compared with vehicle treatment (Lai et al., 2008). Treatment with GCEE also reduced brain tissue loss and death of CA1 and CA3 hippocampal neurons by approximately 30–50% compared with vehicle. The same caveat mentioned above regarding off-target (nonautophagy) related effects of rapamycin also applies to GCEE. However, it can be stated that somewhere between 0 and 50% of cell death and brain tissue loss after TBI in mice is via autophagic neurodegeneration.

Autophagy after hypoxia-ischemia

What is the autophagic burden after hypoxia-ischemia?

The first report of increased autophagy after experimental cerebral hypoxia-ischemia can be credited to Zhu et al. (2005), who also examined the influence of development on baseline and post-ischemic autophagy. Using a model of unilateral carotid artery occlusion and hypoxemia in mice, they reported that PND 5 mice had higher levels of both LC3-I and LC3-II compared with PND 60 mice (Zhu et al., 2005). After unilateral hypoxia-ischemia, relative LC3-II levels were increased above baseline in the ipsilateral hemisphere in PND 5, 9, 21, and 60 mice ($n = 5$ /group), but this increase was only statistically significant in PND 60 mice (~100% over baseline). A separate group, also using a murine model of unilateral carotid occlusion with hypoxemia, reported the presence of vacuole-associated damage via electron microscopy consistent with autophagy in the ischemic brain (Adhami et al., 2006). Using GFP-LC3 transgenic mice, they also showed a qualitative increase in GFP-enriched vesicles at 6 and 18 hours after hypoxia-ischemia. Autophagic neurodegeneration, defined as an increase in LC3-II, has also been reported in a model of transient middle cerebral artery occlusion in adult mice (Degterev et al., 2005) and rats (Liu et al., 2010). However, in the study by Degterev et al. (2005), the authors concluded that the increase in autophagy observed was consequential and not causative of cell death.

In a recent study, Ginet et al. (2009) compared regional differences in autophagy and apoptosis using this model of neonatal hypoxia-ischemia in PND 7 rats. Autophagic flux was assessed by LC3-II, lysosomal enzymes and protein biomarkers such as lysosomal-associated membrane protein 1 (LAMP1); and apoptosis was assessed by caspase-3 activity and other biomarkers (Ginet et al., 2009). These investigators found robust increases in autophagic flux and some correlations with biomarkers of apoptosis that were region-dependent after hypoxia-ischemia. For example, increased autophagy in cerebral cortex was regionally associated with apoptosis, whereas in the CA3 region of the hippocampus it appeared independent of apoptosis. In contrast, apoptosis appeared more prominent than autophagy in the CA1 region of the hippocampus and dentate gyrus (Ginet et al., 2009).

Taken together, there are multiple lines of evidence to suggest that there is a robust increase in autophagy in the ischemic rodent brain. The degree of autophagy triggered by hypoxia-ischemia appears to be brain region dependent. This may be the case in TBI as well;

however, injury in experimental models of TBI is more much more focal than the unilateral carotid artery/hypoxemia model used by Ginet, et al. (2009). The question as to whether increased autophagy is beneficial, detrimental, or an epiphenomenon after hypoxia-ischemia is similar to that posed for TBI—and certainly none clearer.

What is the role of autophagy after hypoxia-ischemia?

Similar to TBI, support for a *beneficial* role of autophagy after cerebral hypoxia-ischemia is based on effects of treatment with rapamycin. Using a pretreatment paradigm, Carloni et al. (2008) administered a single intraventricular dose of the autophagy-inducing agent rapamycin (0.5 ng) 20 min before unilateral carotid artery ligation followed by hypoxemia in PND 7 rat pups. Treatment with rapamycin increased Beclin 1 levels in brain and reduced PI-stained cells by over 50% in the ipsilateral cortex and hippocampus compared with vehicle treatment (Carloni et al., 2008). Further supporting a beneficial role for increased autophagy, pretreatment with nonselective inhibitors of autophagy, 3-methyladenine (3-MA; 5 μ L of a 10 mM solution) and wortmannin, reduced Beclin 1 labeled cells and increased necrotic cell death (Carloni et al., 2008).

In stark contrast to the study described above, is a report by Puyal et al. (2009). These authors reported that a single intracerebroventricular injection of 3-MA (60 μ g) reduced lesion volume by 46% when administered 4 hours after temporary middle cerebral artery occlusion in PND 12 rats (Puyal et al., 2009). While there are certainly differences in terms of pre- versus post-treatment strategy, ischemia model, and a modest difference in age at time of insult, it is difficult to explain the discrepancy in findings. Further confusing the role of autophagy after hypoxia-ischemia is a recent study by Zheng et al. (2009). These authors report that RNA interference (RNAi) knockdown of Beclin 1 reduces autophagosome formation and results in an approximately 50% reduction in infarction volume versus control RNAi after transient middle cerebral artery occlusion in adult rats (Zheng et al., 2009).

We have also begun to characterize autophagy in a model of pediatric cardiac arrest (Fink et al., 2004). This model uses PND 17 rats, again similar to a 1–4 year old child in terms of brain development (Rice and Barone, 2000), and produces asphyxial cardiac arrest. In contrast to adult victims of cardiac arrest, asphyxia (versus dysrhythmia) is the most prevalent cause of cardiac arrest in children (> 80%) (Young et al., 2004). Under an Institutional Animal Care and Use Committee approved protocol, PND 17 rats were subjected to 8.5 min of asphyxial cardiac arrest. Immunohistochemical analysis showed the accumulation of punctate, LC3-positive cells in the CA1 region of the hippocampus (Fig. 3A), the brain region most vulnerable to ischemia in this model (Fink et al., 2004), at 72 hours. Neurodegeneration in CA1 hippocampus identified using fluorojade-C (FJ-C) is shown in Fig. 3B to demonstrate the overlap between regions of neurodegeneration and accumulation of LC3-enriched vesicular structures after ischemia. Neither punctate LC3-positive staining, nor FJ-C staining, is seen in sham controls (data not shown). Ultrastructural analysis showed autophagosomal vacuoles in the CA1 region of the hippocampus, cortex, and cerebellum 24 hours to 7 days after asphyxial cardiac arrest (Fig. 3CF), verifying an autophagic response in brain in this model.

One advantage of cerebral hypoxia-ischemia models over TBI models is the capacity to use very young mice, important when using transgenic mice that develop disease early in life such as *Atg7* deficient mice (Komatsu et al., 2006). A modification of the Levine preparation (Levine, 1960), unilateral carotid artery ligation followed by a period of hypoxemia, can be used in very young rodents (days old) clinically mimicking perinatal asphyxia (Rice et al., 1981). Using this model, Koike et al. (2008) examined the impact of hypoxia-ischemia imposed on postnatal day (PND) 7 in *Atg7* deficient mice. After hypoxia-ischemia, neonatal *Atg7* deficient mice had less hippocampal neuronal death and autophagy compared with

wild-type mice (14 vs. 49%, respectively), implying that autophagy contributes substantially to neurodegeneration after acute cerebral ischemia (Koike et al., 2008). The protective effect of *Atg7* deletion after hypoxia-ischemia could not be determined in older mice, since hippocampal neurodegeneration begins around 3 weeks of age (Komatsu et al., 2006). However, evaluation of the mode of hippocampal neuronal death after hypoxia-ischemia imposed on PND 56 in *wild-type* mice did implicate a role for autophagy (Koike et al., 2008). While off target effects of *Atg7* gene deletion were not examined, these data do suggest that autophagy contributes to ischemic neurodegeneration, perhaps accounting for up to 70% of CA1 hippocampal neuronal death (Koike et al., 2008). This study is the strongest evidence for a detrimental role for autophagy after cerebral ischemia, but may apply only to the developing mouse brain and be model-specific.

Autophagy in other models of acute CNS injury

Increased autophagy has been observed in several other models of CNS injury. After spinal cord injury (SCI), a robust increase in Beclin 1 immunoreactivity is seen in neurons, astrocytes, and oligodendrocytes within the contused spinal cord (Kanno et al., 2009). These authors also found a correlation between Beclin 1 immunoreactivity and TUNEL, leading them to conclude that autophagy contributes to cell death after SCI. In a model of subarachnoid hemorrhage in rats, increased Beclin 1 and cathepsin-D immunoreactivity, in addition to an increased LC3-II/LC3-I ratio, was seen in injured brain (Lee et al., 2009). Obviously, there are many pathophysiologic similarities between SCI, subarachnoid hemorrhage, and TBI.

Autophagic stress is also felt to be a component of excitotoxicity and seizures. A transient increase in LC3-II is observed in the hippocampus in a model of kainic acid-induced excitotoxicity in mice (Shacka et al., 2007). In a model of pilocarpine-induced status epilepticus, increased LC3-II and Beclin 1 are also observed (Cao et al., 2009). These authors also noted that pretreatment with Vitamin E partially suppressed this increase in LC3-II and Beclin 1, thought to be due to the antioxidant properties of Vitamin E.

Discussion

Where does the field stand now?

Two things are irrefutable. First, autophagy is a vital homeostatic process and disruption of autophagy in brain for long durations (as yet unknown, how long) is detrimental. Second, increased autophagy is observed in multiple and distinct experimental models of brain injury including trauma, hypoxia-ischemia, status epilepticus, and subarachnoid hemorrhage. Whether increased autophagy in brain in response to injury is in-and-of-itself a homeostatic response, additive cellular stress (in essence “autophagic stress”), a direct process of neurodegeneration and cell death, or merely a non-contributory consequence of brain damage produced by these insults remains uncertain and controversial (Chu, 2006; Scarlatti et al., 2009; Au et al., 2009).

Approaching a consensus has been hampered by the fact that studies in autophagy-related transgenic mice are confounded by the necessity for some degree of autophagy to maintain homeostasis, and progressive neurodegeneration as the mouse ages. In studies using *Atg7* deficient mice, hypoxia-ischemia was imposed on PND 7, and brains were evaluated 3 days later (Koike et al., 2008), before neuropathology is detectable in these mice around 28 days of life (Komatsu et al., 2006). In addition, the lack of specific pharmacological or molecular agents targeting autophagy cloud interpretation because of issues of off-target effects and/or direct neuroprotective effects of these agents. Rapamycin has been used to augment autophagy after both ischemic and TBI. After TBI, rapamycin further increased autophagy

and improved neurological outcome (Erlich et al., 2007). When administered before hypoxia-ischemia, rapamycin also augmented autophagy and improved neurological outcome (Carlioni et al., 2008). Given that rapamycin also has immunosuppressive and anti-inflammatory effects, and effects on protein synthesis and cell proliferation (Sehgal et al., 1975), it is possible that rapamycin's effects are only partially dependent or independent of autophagy.

Studies testing what is regarded as a more selective inhibitor of autophagy, the class III PI3-K inhibitor 3-MA (Seglen and Gordon, 1982), have actually come to divergent conclusions in related models of hypoxia-ischemia. Post-treatment with 3-MA reduced autophagy and infarction size compared with vehicle treatment in a rat model of transient focal cerebral ischemia in PND 12 rats (Puyal et al., 2009), *i.e. inhibiting autophagy was beneficial*. In contrast, pretreatment with 3-MA reduced autophagy but increased cell death in a rat model of unilateral carotid artery ligation followed by hypoxemia in PND 7 rat pups (Carlioni et al., 2008), *i.e. inhibiting autophagy was detrimental*. Although 3-MA has other effects including inhibition of non-class III PI3-kinases and promotion of glycogen breakdown in hepatocytes (Caro et al., 1988), it is difficult to reconcile why 3-MA would have divergent effects after cerebral ischemia, albeit in subtly different experimental models. In support of a beneficial effect of inhibiting autophagy after hypoxia ischemia, RNAi knockdown of Beclin 1 reduced infarction volume after transient middle cerebral artery occlusion in adult rats compared with control (Zheng et al., 2009).

How do we reconcile the divergent experimental data?

It is possible that the circumstances of manipulating autophagy after brain injury are dependent upon the cell's capacity to respond in relation to the cumulative burden of damaged or dysfunctional macromolecules and organelles. Although baseline autophagy in neurons is low (indeed rarely detected), it is apparent that neurons retain the capacity to increase autophagy in response to stress (Du et al., 2009). Nutrient deprivation in this instance should increase autophagy without necessarily increasing the number of dysfunctional substrates, as the process is intended to increase nutrient sources (Fig. 1 and 4). This is unless of course the cell reaches a critical degree of nutrient deprivation, *i.e.* starvation. In the case of neurodegenerative diseases such as Alzheimer's or Parkinson's disease, or disrupted autophagy, the autophagic burden exceeds the capacity; in the former due to increased accumulation of dysfunctional and/or toxic protein aggregates, in the latter due to markedly impaired capacity (Fig. 1 and 4).

For TBI and cerebral ischemia, boiling the role of autophagy down to "supply and demand" is grossly oversimplified (indeed this may be the case with the above as well). Specifically, TBI and cerebral ischemia results in damage to proteins, lipids, and organelles secondary to activation of proteases and lipases, free radical damage, and a host of other mechanisms (Liou et al., 2003; Zhang et al., 2005). In this case, not only is autophagy increased ("supply"), but the cellular burden of damaged and/or dysfunctional macromolecules and organelles is increased as well ("demand"; Fig. 1 and 4). Thus, the role of autophagy may be dictated by whether or not it can meet intracellular demands. For example, if the increase in autophagic capacity is insufficient, then a build up of dysfunctional macromolecules and organelles occurs. In this case augmenting autophagy (e.g. using rapamycin) would likely be beneficial (Fig. 1 and 4). If on-the-other-hand, the increase in autophagic capacity is in excess, then this may result in consumption of functional macromolecules and organelles. In this case inhibiting autophagy (e.g. using 3-MA) may be beneficial (Fig. 1 and 4). Alternatively, if the increase in autophagic capacity *matches* autophagic burden after brain injury, influencing autophagy one way or the other may be inconsequential or disturb homeostasis, and if left alone, the cell would return to its basal state once the increased autophagic burden was cleared (Fig. 1). In either instance, a logical and effective therapeutic

option is to reduce the autophagic burden after brain injury (e.g. using antioxidants; Fig. 4) (Lai et al., 2008).

Conclusions

A number of distinct experimental studies have demonstrated the activation of autophagic pathways and increased autophagy in response to acute brain injury. These studies have also “muddled the waters” in terms of deciphering the role of autophagy after acute brain injury, and the impact of manipulating the autophagic process. Beyond the scope of this review are issues such as the fate of recycled amino acids and fatty acids, whether the role of autophagy is celltype dependent in brain, and cross-talk and/or overlap between autophagy and apoptosis (Maiuri et al., 2007). Addressing these questions should provide plenty of future “food for thought”, for those studying autophagy and acute brain injury.

Acknowledgments

Supported by National Institute of Neurologic Diseases and Stroke grant R01 NS38620, and National Institute Child Health and Human Development grants T32 HD40686 and R01 HD045968. We’d also like to thank Henry Alexander and Christina Hosler for expert technical assistance.

References

- Adhami F, et al. Cerebral ischemia-hypoxia induces intravascular coagulation and autophagy. *Am J Pathol.* 2006; 169:566–83. [PubMed: 16877357]
- Alirezaei M, et al. Short-term fasting induces profound neuronal autophagy. *Autophagy.* 2010; 6:702–710. [PubMed: 20534972]
- Au AK, et al. Evaluation of autophagy using mouse models of brain injury. *Biochim Biophys Acta.* 2009
- Bayir H, et al. Selective early cardiolipin peroxidation after traumatic brain injury: an oxidative lipidomics analysis. *Ann Neurol.* 2007; 62:154–69. [PubMed: 17685468]
- Boland B, Nixon RA. Neuronal macroautophagy: from development to degeneration. *Mol Aspects Med.* 2006; 27:503–19. [PubMed: 16999991]
- Cao L, et al. Autophagy is upregulated in rats with status epilepticus and partly inhibited by Vitamin E. *Biochem Biophys Res Commun.* 2009; 379:949–53. [PubMed: 19138675]
- Carloni S, et al. Protective role of autophagy in neonatal hypoxia-ischemia induced brain injury. *Neurobiol Dis.* 2008
- Caro LH, et al. 3-Methyladenine, an inhibitor of autophagy, has multiple effects on metabolism. *Eur J Biochem.* 1988; 175:325–9. [PubMed: 3402459]
- Chu CT. Autophagic stress in neuronal injury and disease. *J Neuropathol Exp Neurol.* 2006; 65:423–32. [PubMed: 16772866]
- Chu CT, et al. Autophagy in neurite injury and neurodegeneration: in vitro and in vivo models. *Methods Enzymol.* 2009; 453:217–49. [PubMed: 19216909]
- Clark RS, et al. Autophagy is increased in mice after traumatic brain injury and is detectable in human brain after trauma and critical illness. *Autophagy.* 2008; 4:88–90. [PubMed: 17957135]
- Clark RS, et al. Early neuropathologic effects of mild or moderate hypoxemia after controlled cortical impact injury in rats. *J Neurotrauma.* 1997; 14:179–89. [PubMed: 9151767]
- Davis LM, et al. Fasting is neuroprotective following traumatic brain injury. *J Neurosci Res.* 2008; 86:1812–22. [PubMed: 18241053]
- Degterev A, et al. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat Chem Biol.* 2005; 1:112–9. [PubMed: 16408008]
- Diskin T, et al. Closed head injury induces upregulation of Beclin 1 at the cortical site of injury. *J Neurotrauma.* 2005; 22:750–62. [PubMed: 16004578]
- Du L, et al. Starving neurons show sex difference in autophagy. *J Biol Chem.* 2009; 284:2383–96. [PubMed: 19036730]

- Erlich S, et al. Rapamycin is a neuroprotective treatment for traumatic brain injury. *Neurobiol Dis.* 2007; 26:86–93. [PubMed: 17270455]
- Fink EL, et al. An experimental model of pediatric asphyxial cardiopulmonary arrest in rats. *Pediatr Crit Care Med.* 2004; 5:139–144. [PubMed: 14987343]
- Ginet V, et al. Enhancement of autophagic flux after neonatal cerebral hypoxia-ischemia and its region-specific relationship to apoptotic mechanisms. *Am J Pathol.* 2009; 175:1962–74. [PubMed: 19815706]
- Goedert M, Jakes R. Mutations causing neurodegenerative tauopathies. *Biochim Biophys Acta.* 2005; 1739:240–50. [PubMed: 15615642]
- Ikonomovic MD, et al. Alzheimer's pathology in human temporal cortex surgically excised after severe brain injury. *Exp Neurol.* 2004; 190:192–203. [PubMed: 15473992]
- Jellinger KA. Traumatic brain injury as a risk factor for Alzheimer's disease. *J Neurol Neurosurg Psychiatry.* 2004; 75:511–2. [PubMed: 14966185]
- Kabeya Y, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J.* 2000; 19:5720–8. [PubMed: 11060023]
- Kanno H, et al. Spinal cord injury induces upregulation of Beclin 1 and promotes autophagic cell death. *Neurobiol Dis.* 2009; 33:143–8. [PubMed: 18948195]
- Klionsky DJ, et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy.* 2008; 4:151–75. [PubMed: 18188003]
- Koike M, et al. Inhibition of autophagy prevents hippocampal pyramidal neuron death after hypoxic-ischemic injury. *Am J Pathol.* 2008; 172:454–69. [PubMed: 18187572]
- Komatsu M, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature.* 2006; 441:880–4. [PubMed: 16625205]
- Komatsu M, et al. Impairment of starvation-induced and constitutive autophagy in Atg7- deficient mice. *J Cell Biol.* 2005; 169:425–34. [PubMed: 15866887]
- Lai Y, et al. Autophagy is increased after traumatic brain injury in mice and is partially inhibited by the antioxidant gamma-glutamylcysteinyl ethyl ester. *J Cereb Blood Flow Metab.* 2008; 28:540–50. [PubMed: 17786151]
- Lee JY, et al. Activated autophagy pathway in experimental subarachnoid hemorrhage. *Brain Res.* 2009; 1287:126–35. [PubMed: 19538949]
- Levine S. Anoxic-ischemic encephalopathy in rats. *Am J Pathol.* 1960; 36:1–17. [PubMed: 14416289]
- Liou AK, et al. To die or not to die for neurons in ischemia, traumatic brain injury and epilepsy: a review on the stress-activated signaling pathways and apoptotic pathways. *Prog Neurobiol.* 2003; 69:103–42. [PubMed: 12684068]
- Liu C, et al. Autophagy and protein aggregation after brain ischemia. *J Neurochem.* 2010
- Liu CL, et al. Changes in autophagy after traumatic brain injury. *J Cereb Blood Flow Metab.* 2008; 28:674–83. [PubMed: 18059433]
- Maiuri MC, et al. Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol.* 2007; 8:741–52. [PubMed: 17717517]
- Mizushima N, et al. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Mol Biol Cell.* 2004; 15:1101–11. [PubMed: 14699058]
- Morishima Y, et al. Beta-amyloid induces neuronal apoptosis via a mechanism that involves the c-Jun N-terminal kinase pathway and the induction of Fas ligand. *J Neurosci.* 2001; 21:7551–60. [PubMed: 11567045]
- Puyal J, et al. Postischemic treatment of neonatal cerebral ischemia should target autophagy. *Ann Neurol.* 2009
- Rice D, Barone S Jr. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environ Health Perspect.* 2000; 108(Suppl 3):511–33. [PubMed: 10852851]
- Rice JE 3rd, et al. The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Ann Neurol.* 1981; 9:131–41. [PubMed: 7235629]

- Sadasivan S, et al. Changes in autophagy proteins in a rat model of controlled cortical impact induced brain injury. *Biochem Biophys Res Commun*. 2008; 373:478–81. [PubMed: 18486600]
- Scarlatti F, et al. Does autophagy have a license to kill mammalian cells? *Cell Death Differ*. 2009; 16:12–20. [PubMed: 18600232]
- Scherz-Shouval R, et al. Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. *EMBO J*. 2007; 26:1749–60. [PubMed: 17347651]
- Seglen PO, Gordon PB. 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. *Proc Natl Acad Sci U S A*. 1982; 79:1889–92. [PubMed: 6952238]
- Sehgal SN, et al. Rapamycin (AY-22,989), a new antifungal antibiotic. II. Fermentation, isolation and characterization. *J Antibiot (Tokyo)*. 1975; 28:727–32. [PubMed: 1102509]
- Shacka JJ, et al. Kainic acid induces early and transient autophagic stress in mouse hippocampus. *Neurosci Lett*. 2007; 414:57–60. [PubMed: 17223264]
- Singh R, et al. Autophagy regulates lipid metabolism. *Nature*. 2009; 458:1131–5. [PubMed: 19339967]
- Todde V, et al. Autophagy: principles and significance in health and disease. *Biochim Biophys Acta*. 2009; 1792:3–13. [PubMed: 19022377]
- Young KD, et al. A prospective, population-based study of the epidemiology and outcome of out-of-hospital pediatric cardiopulmonary arrest. *Pediatrics*. 2004; 114:157–64. [PubMed: 15231922]
- Zhang X, et al. Bench-to-bedside review: Apoptosis/programmed cell death triggered by traumatic brain injury. *Crit Care*. 2005; 9:66–75. [PubMed: 15693986]
- Zhang YB, et al. Autophagy is activated and might protect neurons from degeneration after traumatic brain injury. *Neurosci Bull*. 2008; 24:143–9. [PubMed: 18500386]
- Zheng YQ, et al. RNA interference-mediated downregulation of Beclin1 attenuates cerebral ischemic injury in rats. *Acta Pharmacol Sin*. 2009; 30:919–27. [PubMed: 19574998]
- Zhu C, et al. The influence of age on apoptotic and other mechanisms of cell death after cerebral hypoxia-ischemia. *Cell Death Differ*. 2005; 12:162–76. [PubMed: 15592434]
- Zhu JH, et al. Regulation of autophagy by extracellular signal-regulated protein kinases during 1-methyl-4-phenylpyridinium-induced cell death. *Am J Pathol*. 2007; 170:75–86. [PubMed: 17200184]

Fig. 1.
Hypothetical scenarios representing the potential role(s) for autophagy after acute brain injury (please refer to text for discussion).

Fig. 2.

Autophagy in brain after TBI in male and female PND 17 rats. **A.** Western blot for LC3-I and LC3-II ($n = 3/\text{group}$). Semi-quantification of LC3-II (mean $\pm$ SEM, $*P < 0.05$ vs. control; $^{\dagger}P < 0.05$ vs. male; ANOVA/Tukey's post hoc test). **B.** Confocal images showing LC3 (red) immunohistochemistry in a male and female PND 17 rat 24 h after TBI and naïve controls. The sections were also labeled with the neuronal marker NeuN (green) and the nuclear marker DAPI (blue). An increase in punctate LC3 labeling (arrows within insets) consistent with autophagosome formation was observed in the ipsilateral cortex, CA3 region of the hippocampus, and thalamus 24 h after TBI vs. controls. Size bars = 10 μM , except the left lower panel, which = 20 μM . **C-G.** Electron micrographs from injured brain 24 h after TBI. Numerous autophagosomes, secondary lysosomes and multilamellar bodies are shown (arrows). Ipsilateral cortex from a male rat (**C**), hippocampus from a female rat (**D**), and hippocampi from male rats (**E-F**). Size bars = 500 nm.

Fig. 3.

Autophagy in brain after asphyxial cardiac arrest in a male PND 17 rats. *A and B.* Confocal images showing LC3 (red) and Fluor Jade-C (FJ-C; green) immunohistochemistry at 72 h. The sections were also labeled with the nuclear marker DAPI (blue). An increase in punctate LC3 labeling (inset) consistent with autophagosome formation is shown in a section from the CA1 region of the hippocampus. *C-F.* Electron micrographs from rats 24 h – 7 d after asphyxial cardiac arrest. Autophagosomal vacuoles, secondary lysosomes, and a multilamellar body are shown (arrows). CA1 region of the hippocampus from a male rat at 72 h (*C*), cerebellum from a female rat at 7 d (*D*), hippocampus from a female rat at 24 h (*E*), and cortex from a male rat at 24 h (*F*). Size bars = 500 nm.

Fig. 4. Hypothetical “supply” (capacity) and “demand” (burden) curve for autophagy after acute brain injury and response to various interventions tested in experimental models (please refer to text for discussion; 3-MA, 3-methyladenine; Atg, autophagy-related gene; RNAi, RNA interference).