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Autophagy as a Homeostatic Mechanism in Response to Stress Conditions in the Central Nervous System

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Abstract
Autophagy is considered a major bulk degradation system that helps cells to counteract different intracellular and extracellular stress signals. Several protein complexes integrate multiple signals in order to activate autophagy, which sequesters damaged cellular components and carries them to lysosomes for degradation. This active mechanism is essential to maintain cell homeostasis and particularly in neurons to sustain their viability. Because of their polarized morphology, neurons face special challenges to recycle cellular components through autophagy in dendrites and distal regions of axons. Thus, autophagy is critical in the remodeling of pre- and post-synaptic constituents to sustain neuronal functionality. Under stress conditions, autophagy may play either a cytotoxic or a cytoprotective role. This discrepancy is partly due to the lack of a full characterization of the autophagic process and conclusive evidence to support whether basal autophagy is stimulated or impaired in a particular condition. Moreover, in many studies, only pharmacologic tools have been used to modulate autophagy. Throughout the present review, we go over the literature revealing autophagy induction in the nervous system under diverse stressful conditions, the signaling pathways involved, and its consequences for neuronal homeostasis and survival. We have focused on five particular stress conditions that alter neuronal homeostasis and can induce neuronal death including, starvation, oxidative stress, endoplasmic reticulum (ER) stress, proteotoxic stress, and aging.

Keywords Autophagy · Neurons · Starvation · Oxidative stress · Endoplasmic reticulum stress · Aging

Introduction

Autophagy is an evolutionary conserved mechanism in eukaryotes, considered as the major bulk degradation system where damaged cytoplasmic components are delivered to the lysosome for degradation. Autophagy is also responsible for the turnover and recycling of cytoplasmic materials to produce building blocks and energy to maintain cellular homeostasis [1–3]. Several extracellular and intracellular stimuli promote autophagy representing an essential mechanism by which cells can adapt to stress conditions, particularly to starvation. However, basal levels of autophagy are present in all cells in order to maintain cellular homeostasis and energy balance [4].

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Depending on how cytoplasmic material is delivered to the lysosome, autophagy is divided in three classes: macroautophagy, microautophagy, and chaperone-mediated autophagy. In macroautophagy, an isolation membrane (phagophore) sequesters portions of the cytoplasm, including proteins and organelles, to form a double- or multi-membrane structure named autophagosome. The autophagosome fuses with the lysosome to become an autolysosome and degrades its content. Microautophagy involves the engulfment of small portions of the cytoplasm directly by the lysosome membrane to be subsequently degraded [5]. Finally, chaperone-mediated autophagy is a specific type of autophagy, where substrate proteins are targeted one by one toward the lysosome and then translocated across the lysosomal membrane [6]. Macroautophagy has been mostly investigated and is considered as the major subtype of autophagy; thereby, we will refer to macroautophagy as autophagy.

The Autophagy Machinery

The process of autophagy involves the participation of autophagy-related (ATG) proteins, which are recruited to the

phagophore to form the autophagosome. The canonical formation of the autophagosome involves different steps including induction, autophagosome formation, fusion of the autophagosome with the lysosome, and cargo degradation, followed by the release of breakdown products into the cytosol. ATG proteins contribute to these steps and many of them are essential for a proper autophagy function.

A central inhibitor of autophagy is mTOR, a Ser/Thr protein kinase originally recognized as a target of rapamycin, which is involved in numerous cell processes such as protein synthesis, growth, sensing of environmental nutritional changes, and autophagy. mTOR integrates multiple upstream signals which control autophagic activity. Under nutrient-rich conditions, mTOR activation negatively regulates autophagy through inhibition of the ULK1 complex, composed by ULK1, ATG13, FIP200, and ATG101 [7, 8]. In contrast, upon starvation or rapamycin treatment, mTOR inactivation leads to a stable form of the ULK1 complex located at the phagophore to induce autophagy.

In cells undergoing autophagy, autophagosome formation initiates at phagophore assembly sites (PAS), where double-layered membrane elongates to form a phagophore and then an autophagosome [8]. Different organelles are considered as phagophore membrane sources such as endoplasmic reticulum (ER), mitochondria, and the Golgi complex, where multiple PAS can arise [9]. Phagophore formation initiates after ULK1 activation, which in turn targets class III PtdIns3K complex, containing VPS34, BECN1, ATG14, and VPS15, to produce PtdIns3P [7]. After the initiation of the phagophore formation, the elongation of the phagophore membrane that will ultimately become an autophagosome is regulated by two ubiquitin-like reactions. In the first reaction, ATG12 is activated by ATG7 (E1 ubiquitin-activating enzyme) and transferred to ATG10 (E2 ubiquitin-activating enzyme); after these two subsequent reactions, ATG12 is covalently bound to ATG5 to form the ATG12-ATG5 conjugate. This complex interacts with ATG16L1 resulting in a ternary complex ATG12-ATG5-ATG16L1 [8, 10], which localizes to the phagophore membrane and is essential for its elongation. This complex dissociates when the autophagosome is fully formed. The second ubiquitin-like reaction involves the conjugation of ATG8 family, which includes the microtubule-associated protein light chain 3 (MAP-LC3/ATG8/LC3), γ-amino-butyric acid receptor-associated protein (GABARAP), and Golgi-associated ATPase enhancer of 16 kDa (GATE-16), to phosphatidylethanolamine (PE). LC3, the best-characterized ATG8 member, is cleaved by ATG4B to produce the cytosolic form LC3-I [11]. LC3-I is conjugated to PE at the autophagosome membrane through two ubiquitin-like reactions involving ATG7 (E1 like) and ATG3 (E2 like), to form autophagosome-associated LC3-II. The ATG12-ATG5-ATG16L complex regulates the conjugation of LC3-I to PE acting as an E3-like enzyme, although it is not essential for conjugation to occur [8]. LC3-II is associated to both sides of the autophagosome and regulates its size due to its ability to determine membrane curvature.

Autophagosomes are mobilized toward lysosomes along microtubules through dynein-dependent transport; then, the outer membrane of the autophagosome fuses with the lysosome to form an autolysosome, a process that requires the lysosomal membrane protein LAMP-2 and the small GTPase Rab7 [12]. Soon after the fusion, LC3-II attached to the outer membrane is delipidated by ATG4B to recycle LC3-I [13]. Proper function and integrity of the lysosome are essential for successful fusion to occur. Degradation of the inner membrane and autophagosome content including LC3-II is dependent on lysosomal hydrolases such as cathepsin B, D, and L. Degraded autolysosome content is released into the cytosol for protein synthesis and maintenance of cellular homeostasis.

On the other hand, non-canonical pathways of autophagy have been discovered, whereby autophagosome formation occurs in the absence of some key autophagy proteins such as ATG7, ATG5, LC3, and BECN1. These non-canonical pathways have been identified under certain cellular circumstances and their role in cellular homeostasis is not completely clear [14]. For instance, BECN1-independent autophagy contributes to apoptosis in cortical neurons exposed to the pro-apoptotic agent staurosporine [15]. On the other hand, mouse embryonic fibroblast lacking ATG5 or ATG7 can undergo autophagy when exposed to certain stressors such as etoposide, staurosporine, and even nutrient deprivation. Interestingly, under these conditions, lipidation of LC3 does not occur, suggesting that ubiquitin-like proteins are not required for alternative autophagy. However, some conventional autophagy-related proteins including ULK1, BECN1, and VPS34 are required for alternative autophagy. These findings suggest that under certain stress conditions, an alternative autophagy can occur in order to overcome stress. Although, conventional autophagy is necessary for the clearance of protein aggregates in neurons [16].

Autophagy can be either nonselective or selective. Selectivity is mediated by autophagy receptors that recognize both, the tagged cargos with degradation signals and the autophagosome membrane through the LC3-interacting region [17]. The ubiquitin-binding protein p62/SQSTM1 is the best-characterized autophagy receptor and its degradation into the autolysosome has been widely used as a measure of the autophagic flux.

Autophagy in the CNS

In mammalian adult brain, neurogenesis is limited to selected regions, namely, the dentate gyrus of the hippocampus and the subventricular zone. Thus, there is no turnover of dysfunctional neurons and they ought to survive an entire lifetime. As

post-mitotic cells, neurons rely on autophagy for the maintenance of protein and organelle homeostasis and thus neuronal viability.

The relevance of autophagy as a quality control mechanism in the CNS is evidenced in animal models where essential autophagy genes are genetically suppressed. Mice lacking Atg5 [18], Atg7 [19], and FIP200 [20], specifically in the CNS, show neurological and behavioral defects, including abnormal limb-clasping reflexes, locomotor ataxia, and deficits in motor coordination that result in early death. Deficiency of these autophagy-related genes causes the accumulation of polyubiquitinated proteins in almost all brain regions, forming inclusion bodies that increase in size and number with age [18, 19]. Moreover, animals exhibit axonal degeneration and neuronal loss in the cerebral and cerebellar cortices. These findings highlight the importance of a continuous clearance of cytosolic proteins through basal autophagy, in order to prevent the accumulation of abnormal proteins, which might impair neuronal function.

Autophagy plays a housekeeping role degrading aggregate-prone proteins including polyglutamine-expanded huntingtin (mHTT) and mutant α-synuclein (PD) [21]. In agreement, autophagy dysfunction, leading to the accumulation of pathogenic proteins and autophagy vacuoles, has been reported in neurodegenerative disorders [22]. Conversely, stimulation of autophagy reduces the accumulation of mHTT and α-synuclein, protects against cell death, and reduces behavioral alterations in different animal models of neurodegenerative diseases [23–25].

Besides a housekeeping role, autophagy functions as an adaptive response exerting cytoprotective and anti-inflammatory actions under stress conditions [9]. Pharmacological or genetic inhibition of autophagy diminishes neuronal capacity to respond to nutritional, physical, and chemical insults, suggesting an adaptive role that contributes to reestablish physiological conditions [26]. In contrast, there is also evidence supporting that autophagy can lead to cell death, named autophagic cell death, under certain conditions such as starvation and hypoxia/ischemia [27]. Thus, autophagy may exert a cytotoxic rather than a cytoprotective action under some circumstances [28]. The differential role of autophagy as an adaptive response or a mechanism contributing to cell death is related to the intensity and the duration of the stress stimulus (Fig. 1).

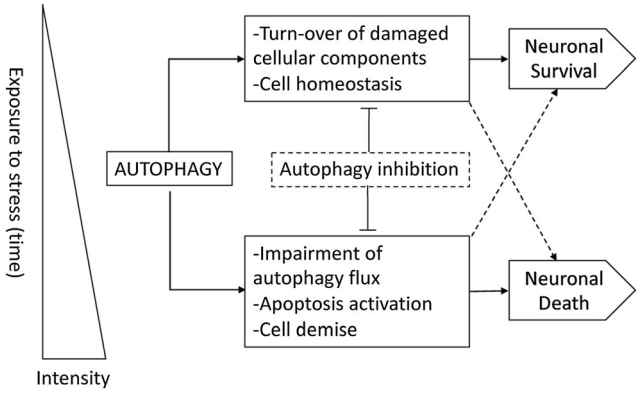

Fig. 1 Stress stimuli induce autophagy that depending on the duration and intensity can elicit different responses. Mild and short stress induce autophagy-dependent turnover of damaged cellular components to restore cell homeostasis that ultimately promote neuronal survival. Prolonged and intense stress induce autophagy dysfunction and apoptosis activation, which in turn causes neuronal death. Inhibition of neuroprotective autophagy leads to accumulation of damaged cellular components and cell homeostasis disruption. By contrast, inhibition of neurotoxic autophagy prevents apoptosis activation and autophagy-dependent cell demise

Autophagy Takes Place in Specific Neuronal Compartments

Neurons are polarized cells with unique morphology. The axon is a specialized compartment that can reach up long distances from the soma, the main site of protein degradation and synthesis. How autophagy regulates protein homeostasis along the different cell compartments is a challenging question [29]. Live-cell imaging studies indicate that autophagosomes are formed at the distal end of the axon. Autophagy core proteins are recruited to ER membranes present at the distal axon, where autophagosomes are generated [27, 28]. Then, autophagosomes travel by retrograde transport toward the soma and fuse with late endosomes or lysosomes, forming autolysosomes as they reach the soma [30, 31]. This process regulates axonal homeostasis and maintains the quality control of proteins present in nerve terminals. On the other hand, in the case of dendrites, it has been suggested that autophagosome biogenesis can occur locally or autophagy vesicles can be recruited to this compartment in a synaptic activity-dependent manner [32].

Synapses are critical neuronal compartments for brain function. Synaptic activity requires the participation of a large amount of proteins, which are highly susceptible to damage that leads to their accumulation and function impairment. Autophagy-recycling plays a crucial role in removing damaged proteins and maintaining homeostasis also in synapses [33]. In addition, autophagy regulates presynaptic structure and function. It has been reported that autophagy deficiency in dopaminergic neurons results in enhanced dopamine secretion in response to stimulation and accelerated presynaptic recovery due to the lack of the turnover of neurotransmitter vesicles [34]. Conversely, enhanced autophagy induced by rapamycin decreased dopaminergic synaptic vesicle density [35]. This suggests that autophagy regulates neurotransmission through the degradation of synaptic vesicles, altering synaptic terminal volume and the release of neurotransmitter. Also, autophagy is involved in synaptic plasticity. After chemical long-term depression, autophagosomes accumulate within dendrites and spines, and partially contribute to AMPA

receptor degradation. Under these conditions, ATG7 knockdown recovers AMPA protein levels only partially, suggesting the involvement of other degradation systems [36].

Starvation and Nutrient Deprivation

Neurons are highly metabolically active with a limited nutrient storage capacity; thereby, they are particularly sensitive to energy fluctuations. Glucose is the obligatory substrate for adult brain and a continuous supply of glucose and oxygen is required during development for normal brain function. Under prolonged fasting, which can lead to moderate hypoglycemia, or during pathological conditions such as ischemia and severe hypoglycemia, the brain is deprived from glucose. In these conditions, autophagy may be triggered in neurons in order to reestablish the energy status.

Metabolic stimuli that lead to autophagy activation are canonically integrated by the master regulator mTOR. However, depending on the stimuli, the upstream signaling can vary. For instance, growth factor deprivation, such as insulin, activates autophagy through PI3K/Akt signaling and subsequent inhibition of mTOR (reviewed in [37]). On the other hand, amino acid starvation is sensed by Rag GTPases that regulate the inhibition of mTOR and consequently ULK1/2 activation leading to autophagy [38]. Under these conditions, ablation of ULK1/2 completely abrogates autophagy activation [39]. Moreover, autophagy induced by glucose starvation also depends on mTOR-ULK1/2 but the upstream signaling involves AMPK activation [40]. Thus, cells can respond to a variety of metabolic challenges inducing autophagy in order to maintain the energy status. Nonetheless, severe conditions that lead to energy depletion result in autophagy impairment. This is because some autophagy steps are ATP-dependent such as lysosomal v-ATPase functioning, which is essential for the autophagosome-lysosome fusion and autolysosome degradation [41].

It is well known that food restriction induces autophagy in many organs. However, pioneer studies in mice exposed to food deprivation during 48 h showed no increase in brain autophagy [42]. The authors hypothesized that under these conditions, other organs could supply the brain with nutrients such as glucose and ketone bodies. However, more recent studies revealed that short-term fasting induces the upregulation of autophagy in cortical neurons and Purkinje cells, mediated by the decreased activation of neuronal mTOR in vivo [43]. In hypothalamic neurons, autophagy is induced by free fatty acids under fasting conditions in order to modulate food intake and long-term energy homeostasis [44]. Inhibition of hypothalamic autophagy alters energy balance and potentiates the development of metabolic diseases. Thus, autophagy within hypothalamic neurons is crucial for the control of food intake, energy and body weight balance [45].

Data on autophagy activation during nutrient stress in cultured neurons are still controversial. It has been observed that deprivation of growth factors and hormones in cortical cultures activates autophagy via mTOR inactivation [46]. In this condition, activation of autophagy protected neurons against proteotoxicity induced by polyQ-expanded huntingtin, suggesting an adaptive role. Conversely, in hippocampal-cultured neurons, nutrient or glucose deprivation markedly decreased mTOR signaling but it was not sufficient to activate autophagy. However, glucose deprivation attenuates retrograde transport of autophagosomes due to ATP depletion [47]. The authors hypothesized that autophagy has a key function regulating neuronal homeostasis, rather than providing amino acids in response to starvation. Likewise, we have recently demonstrated the impairment of the autophagy flux and the accumulation of autophagosomes in response to glucose deprivation in cortical-cultured neurons, due to lysosomal damage [48]. However, the autophagy flux can be restored upon glucose replenishment or when an alternative fuel such as the ketone body beta-hydroxybutyrate is added [49]. Similarly, long-term exposure to glucose deprivation during 18 h induces the accumulation of autophagy markers in the mouse neuroblastoma cell line Neuro2a suggesting the impairment of the autophagy flux [50].

Several molecules regulate autophagy under nutrient-deficient conditions, AMPK is one of the best characterized. AMPK monitors the energy homeostasis of the cells by sensing the AMP/ATP ratio. In addition, different upstream kinases can regulate AMPK activation including, LKB1 (liver kinase B1), CaMKKB (calcium/calmodulin kinase kinase B), and TAK-1 (TGFB-activated kinase-1) [51]. Autophagy is induced by AMPK-mediated mTOR inhibition, via phosphorylation of TSC2 and Raptor; however, AMPK can directly phosphorylate ULK1 and initiate autophagy [52] (Fig. 2a). Hypothalamic AMPK integrates diverse hormonal signals and plays an important role in the regulation of food intake. In hypothalamic cell lines exposed to low glucose or in animals treated with 2DG, autophagy is induced via AMPK activation, which increases the expression of NPY and decreases that of POMC stimulating food intake. Conversely, knockdown of AMPK within the hypothalamus induces the opposite changes in NPY and POMC expression leading to a reduction in food intake and body weight [53].

Under more severe conditions such as cerebral ischemia, morphological and biochemical markers of autophagy have been observed in several studies (reviewed [54]). However, whether autophagy plays a neuroprotective or neurotoxic role is still controversial [55]. Particularly, in ischemic injury, AMPK leads to autophagy activation, and inhibition of the AMPK-autophagy pathway confers protection against brain damage through the restoration of mTOR activity [56]. In contrast, it has been reported that metformin preconditioning

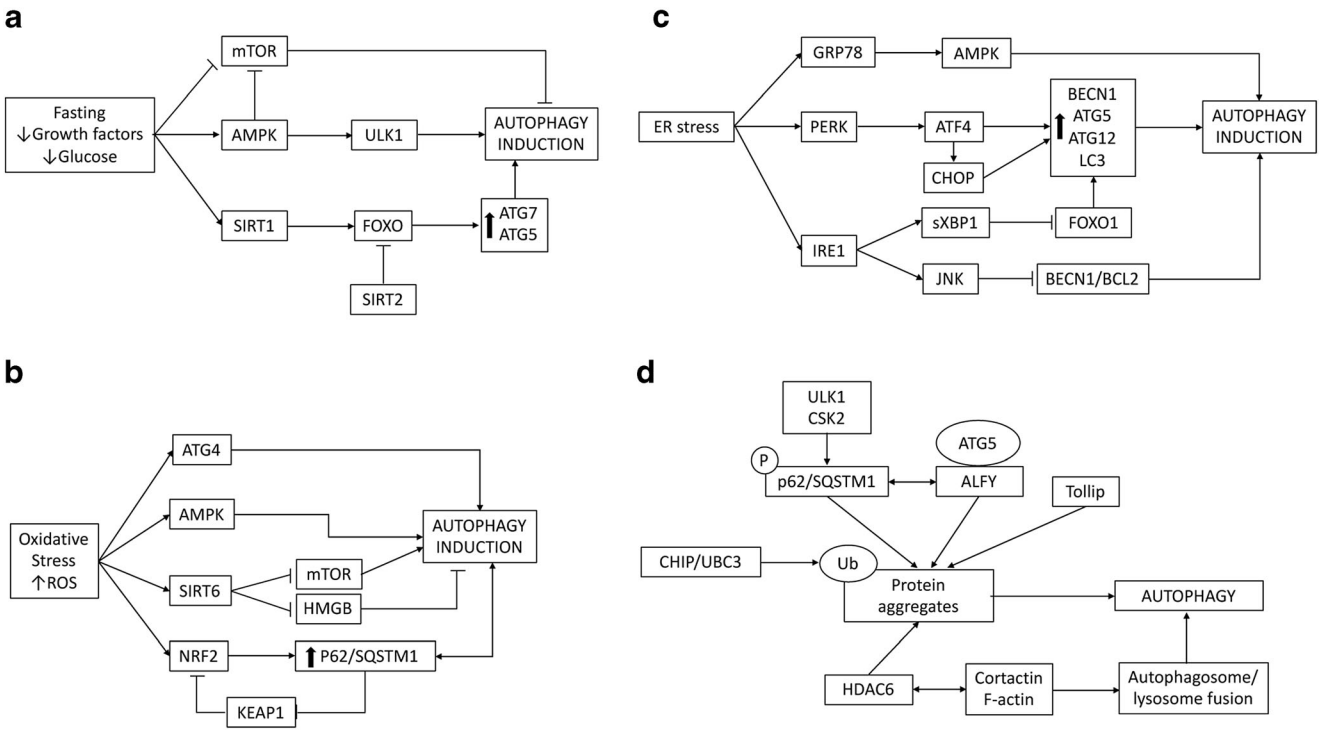

Fig. 2 Signal transduction pathways that regulate neuronal autophagy in response to **a** starvation and nutrient deprivation, **b** oxidative stress, **c** endoplasmic reticular stress, and **d** proteotoxic stress

protects against ischemia by activation of AMPK-dependent autophagy [57]. As mentioned before, autophagy plays an adaptive role in response to different types of stress; however, autophagy defects can compromise this adaptive response increasing the susceptibility to acute stress.

Manipulations that induce autophagy can elicit a hormetic effect increasing the resistance to several stressful conditions [28]. Autophagy induction during ischemic/hypoxic preconditioning exerts protection against a subsequent lethal hypoxic/ischemic insult. The mechanisms involved are not completely clear but some have been proposed. A recent study suggests that autophagy induction by hypoxic preconditioning (HPC) is mediated by the increased expression of HIF-1α and its downstream targets BECN1 and BNIP3, in SH-SY5Y cells exposed to oxygen and glucose deprivation exerting neuroprotection [58]. BNIP3 activates autophagy by mTOR inhibition and through the dissociation of BECN1 form Bcl-2 or Bcl-XL by competition with BECN1, favoring autophagosome formation [59]. Another recent study showed that HPC prevents neuronal damage in the hippocampus of rats exposed to transient global ischemia, through the restoration of the otherwise impaired autophagic flux. This effect involves the stimulation of autophagosome maturation by the activation of the small GTPase, Rab7, mediated by its interaction with UVRAG and Vps16, favoring the autophagosome-lysosome fusion [60].

On the other hand, autophagy activation by rapamycin increases the expression of p-PI3K, p-Akt1, and p-CREB, which correlates with decreased β-amyloid toxicity and improved cognitive decline in rats, suggesting that autophagy is part of a pro-survival response involving the activation of the pAkt1/CREB signaling pathway [61]. The molecular mechanisms involved in the activation of this survival pathway after autophagy induction have not been described. Additionally, rapamycin treatment prior to hypoxia-ischemia promotes neuroprotection through activation of either autophagy or Akt/CREB pathway [62].

Increased tolerance to lethal ischemic injury after ischemic preconditioning (IPC) has been shown to involve the increased expression of GRP78, an endoplasmic reticulum (ER) chaperone implicated in the unfolded protein response (UPR). GRP78 stimulates autophagy through the upregulation of AMPK in in vitro ischemia models, but the molecular mechanisms involved are still not clear [63, 64]. In addition, GRP78 can bind to p62/SQSTM1 to induce a conformational change which favors cargo degradation [65]. Conversely, autophagy activation during IPC upregulates GRP78, HSP60, and HSP70 and reduces the severity of ER stress triggered by lethal ischemia preventing ER-mediated apoptosis. Moreover, rapamycin administration before ischemia exerts similar effects, suggesting that protection elicited by IPC is mediated by the reduction of ER stress through autophagy activation [66]. From these studies, it can be concluded that moderate autophagy activation and mild ER stress induction are responsible at least in part of IPC protection against lethal ischemia.

Autophagy can be also regulated by Sirtuins, a family of NAD-dependent protein deacetylases that sense environmental stress. Sirt1 activity is necessary for the induction of autophagy by starvation, as it interacts and deacetylates autophagy-related proteins including ATG7, ATG5, and LC3 [67]. The lack of Sirt1 results in acetylated ATGs which associates with impaired autophagy, including the accumulation of p62/SQSTM1 and damaged organelles, disruption of energy homeostasis, and early death after birth [68]. Moreover, overexpression of Sirt1 is sufficient to stimulate autophagy even under nutrient-rich conditions. Increasing Sirt1 activity confers protection against axonopathy and neurodegeneration [69]. Furthermore, the sirtuin activator, resveratrol, rescues neuronal dysfunction induced by polyQ and β-amyloid toxicity through autophagy induction [70, 71]. Additionally, sirtuins can enhance the expression of autophagy proteins via deacetylation of FoxO, a well-known transcription factor regulating the expression of autophagy genes (Fig. 2a). Sirt1 promotes FoxO transcription activity, while Sirt2 prevents the interaction of acetylated FoxO with ATG7 preventing autophagy [67]. In addition, neurons lacking Sirt2 showed increased ATG5 acetylation and less recruitment of Parkin toward mitochondria resulting in mitophagy dysfunction [72], suggesting a crucial role of Sirt2 in mitochondrial homeostasis. In contrast, the overexpression of Sirt2 in cholinergic SN56 cells and neuroblastoma SH-SY5Y inhibits the autophagy flux leading to the accumulation of protein aggregates [73] by a mechanism that has not yet been elucidated.

Oxidative Stress and Autophagy

Due to its high metabolic energy requirements and rate of oxygen consumption, the brain is prone to reactive oxygen and nitrogen species (ROS/RNS) production and particularly vulnerable to oxidative stress. The antioxidant capacity of the brain to counteract ROS is limited compared to other organs. In addition, brain contains high levels of polyunsaturated fatty acids, which are targets for lipid peroxidation and act as pro-oxidants [74]. Oxidative stress has been implicated in the pathogenesis of neurodegenerative disorders, which are characterized by high levels of ROS [75]. Under these circumstances, autophagy reduces oxidative damage and ROS levels through the removal of pro-oxidant protein aggregates and damaged organelles [76].

ROS act as signaling molecules and modulate various pathways, including autophagy, through the redox regulation of proteins. Starvation triggers the accumulation of ROS, mainly H_2O_2, which leads to the inactivation of ATG4 ensuring the conjugation of LC3 with the autophagosome membrane, an essential step in autophagosome formation [77] (Fig. 2b). Given the role of ROS in autophagy induction, non-enzymatic and enzymatic antioxidants act as natural downregulators of this process.

Oxidative stress leads to autophagy activation and the accumulation of autophagosomes in different types of cells. Mitochondrial ROS, mainly $O_2^{\cdot-}$, induce autophagy mediated by AMPK activation during starvation [78, 79]. Likewise, in U87 glioblastoma cells, inhibitors of mitochondrial complexes I and II induce ROS-mediated autophagy, and SOD2 overexpression markedly reduces autophagosome formation [80]. Autophagy inhibition reduces the toxicity of mitochondrial electron transport chain inhibitors, suggesting that autophagy contributes to cell death. In normal mouse astrocytes, inhibition of mitochondrial complexes fails to produce ROS and to induce autophagy, possibly because of the lower energy requirements of this type of cells as compared to others such as cancer cells [80]. In contrast, in human neuroblastoma cells, SH-SY5Y rotenone (complex I inhibitor) treatment inhibits the autophagic flux and leads to cell death [81], while treatment with resveratrol, a well-known autophagy inducer, partially rescues SH-SY5Y cells from rotenone toxicity [82]. In the same cell line and in primary dopaminergic neurons, cell death induced by mitochondrial complex I inhibitor MPP+ is accompanied by the activation of autophagy mediated by the ERK/MAPK pathway. Under these circumstances, ablation of autophagy or inhibition of ERK signaling decreases MPP+ toxicity, suggesting the contribution of autophagy to cell death [83, 84]. However, manipulations that enhance autophagy and promote the autophagy flux, such as ischemic preconditioning or pharmacological pretreatments, protect against MPP+ toxicity [85–87], suggesting an adaptive role of autophagy under these circumstances.

On the other hand, oxidative stress caused by the direct exposure to H_2O_2 induces the accumulation of autophagosomes in primary cortical cultures and the genetic or pharmacological ablation of autophagy prevents neuronal death [88]. This result suggests that autophagy contributes to H_2O_2-induced oxidative stress and neuronal death. Furthermore, in SH-5YSY neuroblastoma cells, H_2O_2 exposure induces the activation of autophagy and the subsequent lysosome membrane permeabilization causing cell death [89, 90]. Inhibition of autophagy or prevention of lysosome membrane permeabilization protected cells against H_2O_2 damage. In contrast to H_2O_2 exposure, generation of $O_2^{\cdot-}$ by the xanthine + xanthine oxidase system causes neuronal death accompanied by autophagic features. Under these circumstances, ATG7 knockdown delays early cell death progression, suggesting the involvement of other cell death mechanisms in $O_2^{\cdot-}$-mediated oxidative stress damage [91].

Under acute stress such as traumatic brain injury, ischemia/reperfusion, and hypoxia, the excessive production of ROS leads to the loss of cellular homeostasis and autophagy induction [76]. Excessive production of ROS from NADPH oxidase and autophagy play an important role in neuronal death

induced by hypoxia-ischemia and ischemia/reperfusion in vitro and in vivo [92]. Treatment with the autophagy inhibitor 3-MA effectively reduces the abundance of autophagy markers and OGD-induced neuronal death. Moreover, inhibition of NADPH oxidase decreased autophagy in an OGD model, suggesting that oxidative stress-dependent autophagy, mediated by NADPH, contributes to brain injury [93]. On the other hand, during hypoxic conditions, autophagy can also be regulated by the hypoxia-inducible factor-1 (HIF1), a key player in the cellular response to hypoxia. HIF is stabilized by ROS and activates target genes such as BNIP3 and NIX [94]. In addition, BNIP3 and NIX are involved in mitophagy [95]. However, the direct activation of NIX-mediated mitophagy by ROS has not been explored.

The nuclear transcription factor NRF2 is considered as the master regulator of the antioxidant cell response [96]. NRF2 controls over 250 genes, including p62/SQSTM1, which in turn positively regulates NRF2. Under basal conditions, NRF2 is repressed by Keap1, which mediates its proteasomal degradation. However, ROS-induced autophagy leads to p62/SQSTM1 accumulation and sequestration of Keap1, leading to increased NRF2 signaling [97] (Fig. 2b).

Analysis of postmortem brain tissue from Alzheimer's patients supports the role of p62/SQSTM1 in NRF2 regulation during neurodegeneration. These samples show p62/SQSTM1 accumulation and co-localization with Keap1, suggesting that Keap1 is recruited to autophagosomes allowing NRF2 activity [98]. However, it is well known that autophagy is impaired in neurodegenerative diseases and thereby contributes to neuronal demise; thus, it is unknown to what extent autophagic degradation of Keap1 contributes to NRF2 transcriptional activity in Alzheimer's disease.

On the other hand, SIRT6, a NAD-dependent histone deacetylase, has been implicated in the regulation of metabolism, inflammation, and aging. SIRT6 deficiency leads to oxidative stress and mediates premature senescence-like phenotype, suggesting that disturbances in SIRT6 levels are involved in the aging process. Moreover, SIRT6 counteracts oxidative stress by transactivation of antioxidant genes like NRF2 [99]. SIRT6 stimulates the autophagy flux via attenuation of Akt/mTOR signaling under H_2O_2-induced oxidative stress (Fig. 2b). However, under these circumstances, inhibition of SIRT6 increases neuronal survival by suppressing autophagy [100].

On the other hand, SIRT6 can also modulate autophagy after cerebral ischemia. Reduced SIRT6 levels associate with the release of the high-mobility group box (HMGB1), a chromatin-associated nuclear protein that plays a pivotal function in inflammation and acts as autophagy effector [101]. HMGB1 interacts with BECN1 leading to its subsequent dissociation from Bcl-2 and autophagy activation under oxidative stress [102]. The mitochondrial inhibitor 3-nitropropionic acid (3-NP) induces oxidative stress, autophagy, and striatal neurodegeneration in vivo through HMGB1 activation. Pharmacological inhibition and shRNA targeting of HMGB1 reduces autophagy markers and striatal damage suggesting that HMGB1 is an important signaling molecule for autophagy in neurodegeneration induced by mitochondrial dysfunction [103]. During acute brain damage, HMGB1 is released from neurons and contributes to the inflammatory response. Particularly, after ischemia/reperfusion, HMGB1 mediates brain inflammatory injury and blocking HMGB1 function reverses the inflammatory response and brain damage [104]. Therefore, HMGB1 can be another mediator of oxidative stress-induced autophagy (Fig. 2b).

Altogether, the observations described above suggest that in response to ROS production and oxidative stress, autophagy plays a critical role in the maintenance of cellular homeostasis by degrading damaged proteins and organelles. Several molecules and proteins integrate ROS signals leading to autophagy activation. However, the correlation of autophagy markers with neuronal death under oxidative stress conditions suggests its contribution to neuronal damage, due to the impairment of this process, such as that resulting from the interruption of the autophagic flux. Therefore, genetic of pharmacologic ablation of autophagy confers protection against damage induced by oxidative stress. On the other hand, manipulations that enhance autophagy or stimulate the autophagy flux increase the resistance to oxidative stress conditions and promote cell survival.

ER Stress, UPR Activation, and Autophagy

The ER is an important organelle involved in cellular homeostasis and protein quality control. Several extracellular or intracellular stimuli can disturb ER homeostasis leading to ER stress. To counteract this condition, cells activate the UPR, which reestablishes ER function and ultimately cell homeostasis. Three ER transmembrane sensors, PERK (PKR-like ER kinase), IRE1 (Inositol-requiring enzyme 1), and ATF6 (activating transcription factor 6), orchestrate the UPR, which attenuates global protein translation, upregulates protein folding capacity, and executes programmed cell death. ER stress has been shown to induce autophagy through UPR signaling. Under these circumstances, autophagy functions as an adaptive response to alleviate ER stress.

The mechanisms involved in autophagy induction by activation of the UPR have not been completely elucidated but some reports have implicated GRP78/Bip. GRP78/Bip is an ER-resident chaperone that binds to and inhibits the three UPR sensors. Accumulation of unfolded proteins within the ER lumen recruits GRP78 releasing UPR sensors and allowing UPR activation. As mentioned above, GRP78 can regulate autophagy during ischemic preconditioning. Also, in primary cortical neurons, it has been reported that GRP78 can regulate autophagy. Exposure to cadmium produces ER stress,

which induces autophagy mediated by GRP78 upregulation. Conversely, GRP78 siRNA blocks the conversion of LC3-I to LC3-II and autophagosome formation. Furthermore, it is suggested that cadmium-induced GRP78 upregulation stimulates autophagy via AMPK activation [105] (Fig. 2c).

Under severe brain injury conditions such as hypoxia/ischemia, ER stress and UPR activation contribute to neuronal death [106]. In addition, the PERK and the IRE1 branches of the UPR can activate autophagy [107]. However, the role of autophagy in ischemic injury is still controversial. During ischemic preconditioning, mild ER stress induces autophagy and confers neuroprotection against the ischemic insult. Therefore, inhibition of ER stress and subsequent autophagy activation inhibits neuroprotection elicited by ischemic preconditioning [106]. Moreover, mild ER stress induced by tunicamycin triggers an autophagic response, which protects against 6-OHDA toxicity in mice [108]. On the other hand, it has been shown in in vitro and in vivo models of ischemia-reperfusion that autophagy is activated during the reperfusion phase contributing to the clearance of damaged mitochondria. In agreement, pharmacological or genetic inhibition of autophagy enhanced neuronal damage [109]. In accordance with these results, tunicamycin and thapsigargin administration during reperfusion confers protection against transient ischemia, enhancing autophagy, particularly mitophagy, through PERK-eIF2a-ATF4 signaling [110] (Fig. 2c).

ER stress and UPR dysfunction play an important role in the pathogenesis of neurodegenerative diseases and aging. In Huntington's disease models, upregulation of IRE1 kinase activity leads to impaired autophagic flux and thereby accumulation of mHTT, while reduction of IRE1 expression or inhibition of its kinase activity reduced the accumulation of mHTT [111]. However, how IRE1 inhibits the autophagy flux still remains to be elucidated.

IRE1 can induce autophagy by recruiting TRAF2/ASK and the activation of JNK, which phosphorylates Bcl-2, disrupting its interaction with BECN1, thereby activating autophagy [112] (Fig. 1c). This signaling depends on IRE1 kinase activity. In streptozotocin-induced diabetic mice, ER stress and UPR lead to cognitive decline. In this condition, autophagy activation through the JNK pathway counteracts ER stress-associated neuronal damage [113]. In addition, IRE1 RNAse activity can inhibit autophagy through the downregulation of FOXO1, a transcription factor that regulates autophagy through the upregulation of ATG5, LC3, and BECN1 transcription [114] (Fig. 2c). It has been shown in a transgenic mice model expressing mHTT that IRE1 RNAse activity leads to XBP-1 alternative splicing, which downregulates FOXO1 promoting its degradation by the proteasome. Thus, XBP1 deficiency enhances FOXO1 expression and autophagy reducing HD neurodegeneration [115]. These results suggest that IRE can induce opposite effects on autophagy activation through either its mRNAse or kinase activity.

The PERK branch of the UPR has been also implicated in autophagy. It has been reported that polyQ aggregates induce the activation of PERK/eIF2α-dependent autophagy, as defense mechanism against polyQ-induced cell death. PERK/eIF2α activates autophagy possibly through the upregulation of ATG12 mRNA and increased ATG5-dependent LC3 lipidation [116]. Furthermore, it is known that ATF4 and CHOP, the downstream transcription factors of the PERK pathway, induce the expression of autophagy genes such as ATG12, ATG5, LC3, and BECN1, connecting UPR signaling and autophagy (Fig. 2c) [117].

Proteotoxic Stress and Autophagy

Neurons are particularly vulnerable to the accumulation of misfolded proteins; due to their post-mitotic nature, protein aggregates cannot be dissipated by cell division. Additionally, the decline of degradation systems with advancing age further contributes to neuronal vulnerability. Approximately 30% of the newly synthetized proteins are misfolded and prone to form aggregates interfering with normal cellular function [118]. Therefore, quality control systems continuously operate to manage the flux of misfolded proteins and maintain proteostasis. This quality control machinery involves refolding (chaperones) and degradation (autophagy and ubiquitin proteasome system). The ubiquitin proteasome system (UPS) represents the first line of defense against misfolded proteins; however, aggregates or oligomeric forms of misfolded proteins are not efficiently degraded by the proteasome. Moreover, accumulation of protein aggregates leads to proteasome inhibition resulting in further accumulation of misfolded proteins [119]. Conversely, autophagy can target large protein complexes, aggregates, and organelles. Upon UPS inhibition, autophagy activity increases as a compensatory mechanism to alleviate subsequent proteotoxic stress [120]. Basal neuronal autophagy is critical to prevent the accumulation of ubiquitinated proteins and inclusion bodies, which can disrupt neuronal function [18, 19]. Thus, any genetic or pharmacologic manipulation that enhance autophagy results in the clearance of a variety of aggregate-prone disease proteins.

As we mentioned before, p62/SQSTM1 is the best-characterized autophagy receptor with a role in the clearance of ubiquitinated proteins, aggregates, and organelles [17]. Additionally, it has been identified as a common component of neuronal protein inclusions in several neurodegenerative diseases [121]. Phosphorylation of p62/SQSTM1 at serine 403 by casein kinase 2 (CSK2) increases the affinity of p62/SQSTM1 for polyubiquitinated proteins and the subsequent autophagy-mediated degradation. Overexpression of CSK2 reduces the formation of mutant huntingtin large inclusions [122]. Moreover, overexpression of mHTT induces the expression of p62/SQSTM1, suggesting that p62 plays an important role

in response to proteotoxicity [123]. Likewise, proteotoxic stress induced by proteasome inhibition or expression of polyQ-HTT causes ULK1-dependent phosphorylation of p62/SQSTM1 at S409. This phosphorylation increases binding affinity of p62 to ubiquitinated proteins. In contrast, phosphorylation of p62/SQSTM1 by ULK1 does not occur under amino acid or glucose deprivation, suggesting that ULK1-p62/SQSTM1 cascade is involved in the sensing of proteotoxic stress [124]. However, the upstream signaling pathway which activates ULK1-p62/SQSTM1 cascade remains unknown.

Another protein involved in the interplay between proteasome and autophagy is the histone deacetylase 6 (HDAC6), a microtubule-associated deacetylase that interacts with polyubiquitinated proteins. In Drosophila, HDAC6 activity is critical for autophagy in order to compensate UPS impairment. Furthermore, ectopic expression of HDAC6 is sufficient to prevent degeneration caused by proteasome inhibition and polyQ toxicity [125]. Additionally, HDAC6 controls the fusion of autophagosome with lysosomes in basal autophagy that targets protein aggregates. HDAC6 recruits and activates an actin-remodeling factor, cortactin, to the autophagy substrates and the subsequent assembly of F-actin network that facilitates the fusion of the autophagosome with the lysosome [126] (Fig. 2d). Interestingly, HDAC6 is not required for starvation-induced autophagy.

These two ubiquitin-binding proteins, p62/SQSTM1 and HDAC6, confer specificity to quality control by autophagy and distinguish aberrant protein aggregates and damaged organelles from normal ones. p62/SQSTM1 and HDAC6 bind preferentially lysine 63 (K63)-linked ubiquitinated proteins. Deletion of p62/SQSTM1 or HDAC6 induces a robust accumulation of K63-linked ubiquitin chains in mice brain and MEFs, respectively. This suggests that the specific type of ubiquitin chains added to misfolded proteins determines to which degradative pathway they are delivered [119]. On the other hand, CHIP, a chaperone-dependent ubiquitin E3 ligase, can form a complex with UBC3, which catalyzes the formation of ubiquitin chains in K63. Thus, this complex can direct the degradation of misfolded proteins via autophagy (Fig. 2d). In addition, CHIP colocalizes with α-synuclein and Hsp70 in Lewy bodies a hallmark of Parkinson disease, suggesting that CHIP plays a role in α-synuclein aggregation and degradation. Overexpression of CHIP promotes the degradation of α-synuclein by both proteasome- and autophagy-dependent mechanisms [127].

ALFY (autophagy-linked FYVE protein) has been shown to colocalize with ubiquitin and p62/SQSTM1-positive aggregates under stress conditions [128]. This protein acts as a scaffold which interacts with p62/SQSTM1 and ATG5 and recruits the autophagy machinery toward protein aggregates (Fig. 2d). ALFY is essential for selective autophagy under proteotoxic stress and its overexpression decreases the accumulation of polyQ-HTT and confers neuroprotection [129]. Conversely, ablation of ALFY in Drosophila results in the accelerated accumulation of ubiquitinated-positive inclusions and neuronal degeneration [130]. Tollip is another ubiquitin-binding protein with a role in the clearance of cytotoxic proteins such as polyQ-HTT. Tollip binds to polyubiquitinated proteins with high affinity compared to p62/SQSTM1 and may function cooperatively with p62/SQSTM1 in selective autophagy [131]. Interestingly, Tollip is downregulated in brain tissue samples from aged and Alzheimer's affected humans [132].

Besides protein aggregates, damaged organelles such as mitochondria are tagged with ubiquitin to trigger selective autophagy (mitophagy). Upon mitochondrial damage, the ubiquitin kinase PINK1 is translocated toward the outer membrane of mitochondria and phosphorylates ubiquitin. This activates the ubiquitin ligase Parkin, which builds ubiquitin chains to recruit autophagy receptors and subsequently the autophagy machinery. Under these conditions, PINK1 recruits the autophagy receptors NDP52 and optineurin but not p62/SQSTM1 [133]. Dysfunctional mitophagy is associated with degeneration in several neurodegenerative disorders (reviewed in [134, 135]).

Aging and Autophagy

Aging is associated with the loss of proteostasis, accumulation of altered macromolecules, and a decreased turnover of cellular components [136]. Reduced protein degradation can result from alterations in the autophagic/lysosomal system that finally contributes to the aging process [137]. In aged tissues, reduced expression of ATG proteins and other proteins required for autophagy induction has been reported. Particularly, in the human aged brain, key autophagy genes such as Atg5, Atg7, and BECN1 are downregulated, suggesting that normal aging is associated with decreased autophagy [138]. As previously mentioned, autophagy plays a protective role against neurodegenerative diseases; hence, its downregulation during aging might contribute to the predisposition to neurodegenerative diseases. Moreover, a decline in synaptic plasticity and deficient memory and cognitive function related to aging can be associated with the progressive transcriptional downregulation of autophagy genes [138, 139]. In addition, several autophagy proteins including ATG5-ATG12 and BECN1 complexes are highly reduced in old murine brains suggesting low levels of basal autophagy [140]. Furthermore, old brain tissue shows a significant increase in the protein level of mTOR, suggesting enhanced mTOR activity, which together with decreased autophagy machinery, may play an important role in the age-related impairment of autophagy [140].

A role of autophagy as an anti-aging mechanism has been suggested. Several interventions that modulate metabolism (augmenting ketone bodies), energy intake (caloric restriction), and energy expenditure (exercise) are shown to enhance

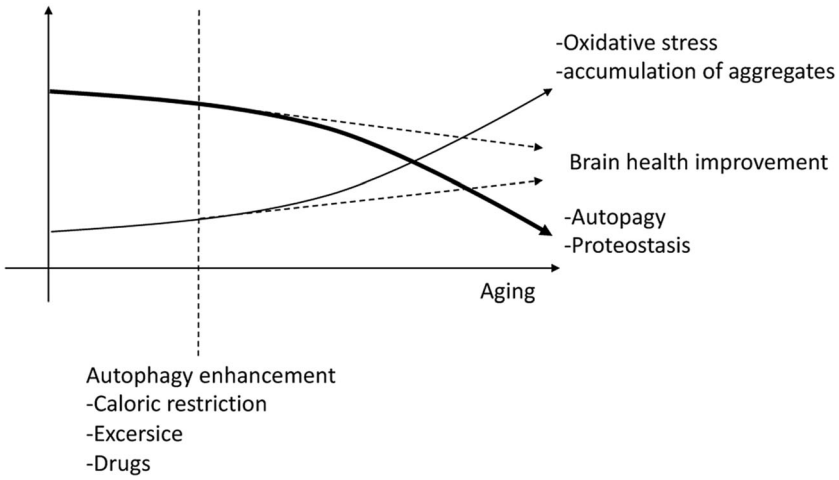

Fig. 3 Autophagy decreases with age leading to loss of proteostasis, accumulation of protein aggregates, and oxidative stress. Any intervention that enhances autophagy results in brain health improvement

autophagy and improve brain health. Inhibition of insulin-like growth factor promotes autophagy and shows an anti-aging effect extending the life span in some species such as the worm *C. elegans* [141]. Moreover, autophagy is required for dietary restriction to extend *C. elegans* life span [142]. Furthermore, caloric restriction delays the onset of age-associated pathologies including brain atrophy and extends life span in the rhesus monkey [143]. Likewise, in rodent models, caloric restriction extends the life span and reduces several age-associated process [144], although it is still not clear whether these effects are mediated by increased autophagy. Long-term exercise training ameliorates cognitive decline in aged rats and reduces oxidative stress. These effects are associated with the activation of autophagy in the hippocampus, which promotes mitochondrial recycling (mitophagy) [145]. Synaptic dysfunction is an early feature of aging that precedes neuronal loss and ultimately neurodegeneration. Therefore, restoration of synaptic homeostasis through the stimulation of autophagy has become a common strategy in order to help neurons to clear damaged proteins or aggregates [33]. Administration of spermidine increases the viability in an autophagy-dependent manner in several types of cells, including neurons [146]. Furthermore, this natural polyamine suppresses memory impairment by counteracting age-dependent synapse dysfunction in Drosophila. Conversely, memory improvement is impaired by deletion of Atg7 and Atg8 autophagy genes [147].

In addition to these findings, it has been observed that skeletal neuromuscular junctions of aged mice display a variety of alterations including axonal swelling and synaptic detachment, associated with autophagy impairment [148, 149]. Moreover, inhibition of autophagy exacerbates neuromuscular synaptic dysfunction and promotes the aging phenotype. Conversely, stimulation of autophagy by caloric restriction or exercise decreases pre- and post-synaptic abnormalities and reduces the loss of motor neurons in aged mice [148]. In agreement, it has been observed that metformin, an AMPK activator and a well-known autophagy inducer, mimics caloric restriction benefits in health. Long-term treatment with metformin counteracts oxidative stress augmenting the antioxidant defense and autophagy, suggesting that metformin promotes healthy aging in the brain [150]. Altogether, these observations lead to the suggestion that impaired autophagy contributes to brain aging and that pharmacologic or nutritional strategies to induce autophagy favor healthy brain aging (Fig. 3).

Conclusions

Autophagy is a major degradation system responsible for the turnover of cytoplasmic material in order to maintain cellular homeostasis. Particularly in neurons, autophagy plays a crucial role in the maintenance of protein and organelle

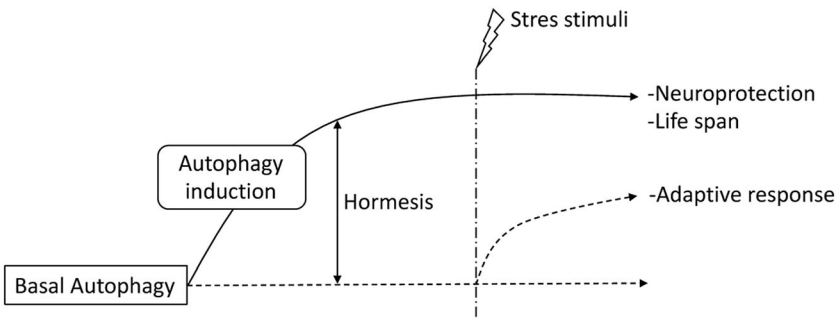

Fig. 4 Autophagy induction in the absence of stress stimuli leads to hormesis. Pharmacologic or nutritional manipulations that induce autophagy increase the resistance to stressful conditions resulting in neuroprotection or extended life span

homeostasis due to their post-mitotic nature. Besides its housekeeping role, autophagy is especially relevant as an adaptive mechanism in response to several stressful conditions to restore homeostasis and promote cell survival. However, autophagy has been implicated in neuronal damage, particularly that associated with acute injury, as autophagy markers correlate with cell death. Hence, ablation of autophagy elicits beneficial effects against brain injury. Nevertheless, in many reports, the lack of evidence to demonstrate whether the accumulation of autophagy markers (i.e., LC3-II, autophagosomes) results from the induction of autophagy or from the impairment of the autophagy flux has made it difficult to conclude about the role of autophagy. Therefore, in order to prove that autophagy is involved in cell death, it is essential to use both pharmacologic and genetic tools to inhibit autophagy components. On the other hand, under severe and prolonged stress insults, autophagy failure such as that induced by lysosome membrane permeabilization can trigger other cell death mechanisms, leading to the misconception that autophagy contributes to cell death. Finally, it is becoming clear that treatments that enhance autophagy (i.e., pharmacologic, nutritional, or preconditioning) or promote the autophagy flux increase resistance to acute stress and extend life span, supporting the idea of autophagy as an adaptive response (Fig. 4).

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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