Unique Features of Neuronal Autophagy: Considerations for Therapeutic Targeting

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Abstract
Autophagy is an essential catabolic pathway responsible for the maintenance of organismal homeostasis. Degradation of damaged organelles and proteinaceous aggregates predominantly takes place via autophagy and a proper function of autophagy is vital for cellular surveillance. Given their post-mitotic nature, neurons are particularly vulnerable to stress and, consequently, robust housekeeping systems are required to guarantee the adequate functionality and viability of neurons. A vast literature links defective autophagic function to neurodegenerative diseases and dietary/pharmacological activation of autophagy has been proposed as potential strategy to fight these diseases. Here we summarize the recent progress on the research of neuronal autophagy highlighting the unique features of autophagy in neurons. In the last section, we discuss about therapeutic strategies modulating autophagy to preserve neuronal surveillance during aging.
Keywords: neuronal autophagy, neuronal homeostasis, mTOR, therapeutic strategies

Autophagy in neuronal homeostasis

Autophagy is a catabolic quality-control process by which intracellular material is targeted to the lysosomal compartment for degradation. Autophagy has been subdivided in three subtypes depending on how the material is delivered to the lysosomes: microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy. Only the latter has been proved efficient in the clearance of aggregates/organelles while the other 2 subtypes, microautophagy and CMA, have been primarily involved in the quality control of cytosolic proteins so far (Bejarano and Cuervo, 2010). Given that the removal of toxic aggregates and organelle recycling is vital for the neuronal surveillance, we will focus the review on macroautophagy (hereafter referred to as autophagy), quantitatively the major autophagic route, the only autophagic pathway highly conserved through the evolution from yeast to mammals and the best-characterized form of autophagy at the molecular level.

Autophagy is responsible for the removal of proteinaceous aggregates and damaged organelles such as mitochondria or peroxisomes through the *de novo* formation of double-membrane organelles called autophagosomes (Fig 1). The synthesis of these double-membrane vesicles is a tightly regulated process that involves the contribution of different autophagy-related proteins (ATGs). The autophagic process can be divided in several steps: activation of autophagosomal biogenesis, isolation of the autophagosomal membrane (initiation), elongation, maturation and, finally, fusion of the mature autophagic vesicles with the lysosomal compartment, which provides the proteases. Every of these steps requires the contribution of specific sets of ATGs. In the last step, these degradative vesicles containing damaged material fuse with the lysosomal compartment to degrade efficiently the cargo inside (Fig.1). In most of the tissues, autophagy is an inducible pathway and the activation of the route depends on the nutritional status. Nonetheless, it is not the only stimulus

activating autophagy thus it has been shown that hypoxia, oxidative stress or acute brain injury also trigger the autophagy process (Galluzzi et al., 2016).

The mammalian target of rapamycin complex 1 (mTORC1) is considered the master regulator of autophagy during starving conditions. mTORC1 blocks autophagy through the inhibition of Unc-51-like kinase (ULK) complex consisting in ULK1/2, Atg13 and FIP200 (Fig.1). When levels of amino acids are low, mTORC1 modifies its distribution from lysosomal to cytosolic location and the mTORC1 complex is not able to inhibit the autophagic process anymore (Bejarano and Rodriguez-Navarro, 2015). In addition to the participation of ULK-Atg13-FIP200 complex in the initiation of autophagosomal biogenesis, other two activities are required for the induction of autophagy: the contribution of Atg9 as lipid donor and the activity of the class III PI3K complex composed by Beclin-1, Vps15, Vsp34 and Atg14. The formation of autophagosomes is thus highly dependent of phosphatidylinositol 3-phosphate synthesis (PI3P) and factors participating in PI3P metabolism regulate the autophagy initiation (Calvo-Garrido et al., 2014; Vergne and Deretic, 2010). Once these three activities converge, the recruitment of the complex Atg16-Atg5-Atg12 occurs triggering the formation of the double-membrane organelle. During the emergence of the double membrane, cytosolic material is engulfed in the vesicles with the participation of different adaptors such as p62 or Optineurin and, finally, a sequential recruitment of Atg8 homologues (LC3, GABARAP and GATE-16) is required for the maturation of the autophagic vesicle before the fusion with the lysosomal compartment (Fig.1).

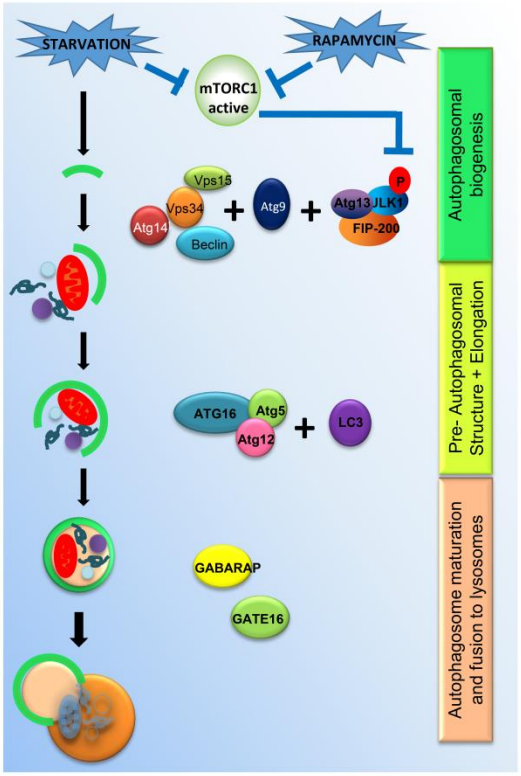

Fig. 1. A schematic representation of the autophagic process. Upon stress, the activities from class III PI 3-kinase complex, ULK-complex and the putative lipid donor Atg9 converge to determine the region from where the isolation membrane emerges. mTORC1 acts as a master regulator of the process by modulating negatively the activity of ULK-complex. Rapamycin mimics cellular starvation by block the mTORC1 activity that does not phosphorylate ULK anymore, triggering the autophagosomal biogenesis. The cargo is recruited and the complex Atg16-Atg5-Atg12 along with the active form of LC3 is responsible for the elongation and closure of the membrane originating the autophagosome. Finally, the autophagosome fuses with the lysosomal compartment that provides proteases for the cargo degradation

Why is autophagy critical in neuronal homeostasis? Most of the neurons differentiate during embryogenesis. They remain in the brain during the whole lifetime and, given that neurons are not dividing cells, the absence of cell division does not allow diluting the accumulation of cytotoxic damage. Consequently, highly

efficient quality-control systems are required to ensure the long-term viability of the neuronal network. Therefore, it is not surprising that the loss of autophagy in the central nervous system causes neuronal cell death and neurodegeneration (Hara et al., 2006; Komatsu et al., 2006). Autophagic malfunction has been identified afterwards in several neurodegenerative diseases including Huntington's, Alzheimer's, Parkinson's or amyotrophic lateral sclerosis (Martinez-Vicente and Cuervo, 2007; Wong and Holzbaur, 2015) and the physiological decline of autophagy with age has been proposed as a potential driver to the late onset of these neurodegenerative disorders (Cuervo, 2003; Kaushik and Cuervo, 2015). In fact, mutations in different autophagy related genes cause neurodegenerative diseases. Some Parkinson disease´s cases are caused by mutations or deletions in PTEN-induced putative kinase protein 1 (PINK1, encoded by PARK6/PINK1) or Parkin (encoded by PARK2/Parkin) both implicated in the selective degradation of damaged mitochondria by autophagy, a process termed "mitophagy". When mitochondria are damaged and lose their membrane potential, mitochondrial PINK1 is stabilized and recruits Parkin, which ubiquitinates a number of mitochondrial membrane proteins, resulting in selective mitophagy (Jiang and Mizushima, 2014). In amyotrophic lateral sclerosis or in frontotemporal dementia, mutations in key adaptor proteins in the autophagic process like p62/sequestosome, Optineurin or ubiquilin2 are suggested to cause genetic forms of these diseases (Deng et al., 2011; Majcher et al., 2015; Maruyama et al., 2010; Rubino et al., 2012; van Blitterswijk et al., 2012). Further research is required for determining the role of these possible pathogenic mutations in these diseases.

Unique features of neuronal autophagy

Although it is out of debate that a proper balance of autophagic function is vital for maintaining neuronal homeostasis, it is only in the last 10 years when the fundamental mechanisms behind neuronal autophagy has been revealed.

Neurons are highly polarized cells with axons that extend long distances and the recycling of organelles/proteins must be modulated in the entire extension, from the soma to the distal processes. Therefore, the neuronal post-mitotic nature makes autophagy an indispensable pathway in neurons and it has been showed that autophagy plays a key role in neuronal development and axonal maintenance (Fimia et al., 2007; Komatsu et al., 2007).

One of the most intriguing unique characteristics of neuronal autophagy is the precise spatio-temporal modulation of the process. Recent reports by using live-cell imaging identified two different populations of autophagosomes in neurons: highly motile autophagosomes derived from the axons (positive for the late endosomal/lysosomal marker LAMP1) that undergo retrograde transport towards the cells body and a second pool of autophagosomes originated directly in the soma (negative for LAMP1) with low motility (Maday and Holzbaur, 2012, 2014, 2016; Maday et al., 2014)(Fig.2). The presence of LAMP1 in autophagosomes from distal axons suggests the early fusion between autophagosomes with late endosomes. In line with this hypothesis, a recent study proposed that fusion events facilitate the recruitment of minus-end motor proteins favoring the retrograde transport (Cheng et al., 2015a, b).

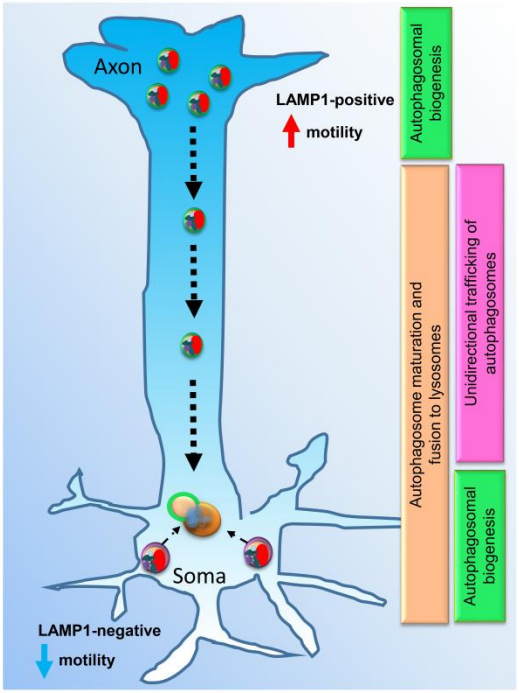

Fig. 2. Unique features of neuronal autophagy. Autophagic vacuoles are generated in the axon and undergo retrograde transport to the soma where the fusion with the lysosomal compartment takes place. These axonal autophagic vacuoles contain LAMP1. By contrary, autophagosomes generated in the soma are LAMP1-negative and the motility is low. In neurodegenerative diseases, aberrant accumulation of autophagosomes takes place in the axons and axon terminals and impairment of autophagy lead to axonal dystrophy

Although the molecular determinants involved in the differential autophagosomal biogenesis within different neuronal compartments remain unclear, these recent reports support the existence of a gradient for autophagic activity from axons to soma. Interestingly, the inhibition of lysosomal proteolysis leads to autophagosomal accumulation exclusively in the soma, not in the axons or dendrites suggesting that the delivery of proteases for the degradation of the cargo takes place entirely in this compartment (Maday and Holzbaur, 2016). Therefore, the soma would act as the primary location for autophagic clearance even when the biogenesis of autophagic vesicles can occur far away (Fig.2).

A fine-tuned regulation is required for the retrograde transport of the autophagic vesicles from the axons to the soma. Nevertheless, the molecular determinants responsible for this one-way vectorial movement are unknown. These studies support a major role for the minus-end motor protein dynein in the dynamics and trafficking of autophagosomes. Additionally, the authors suggest the presence of a one-way sorting mechanism at the base of the axons given that, once the autophagosomes reach the soma, the autophagosomes vesicles remain restricted in the perinuclear area and the re-entry into the axons is not allowed (Maday and Holzbaur, 2014, 2016).

In addition to the spatio-temporal regulation of autophagy in neurons, the most controversial aspect of neuronal autophagy is probably the low ability of neurons to upregulate the autophagic activity upon stress. The most well-known stimulus activating autophagy is the deprivation on nutrients and, although nutrient deprivation leads to activation of autophagy in multiple tissues, no effects were observed in brain by using a mouse model overexpressing a GFP-tagged autophagic marker that allows the tracking of autophagic vacuoles (Mizushima et al., 2004). Again, it is not surprising because, except in extreme circumstances of nutrient deprivation, other tissues could supply nutrients to maintain the functionality of the privileged neuronal tissues. Interestingly, it was reported that short-term fasting is sufficient to induce robust autophagic activity *in vivo*, at least in cortical neurons and Purkinje cells (Alirezaei et al., 2010). *In vitro* studies showed that long-term periods of nutrient starvation are required to upregulate autophagy in

neurons (Boland et al., 2008) and the most recent study reported that starvation is not sufficient to upregulate autophagy in hippocampal neurons (Maday and Holzbaur, 2016) suggesting that unknown factors could be modulating the autophagic activity in neurons upon other type of insults. Therefore, nutritional stress can be a less efficient method to modulate neuroautophagy due to the mobilization of energy sources in response to starvation in other tissues.

Moreover, the autophagic process exhibits a robust constitutive activity even in basal conditions (Maday and Holzbaur, 2012), contributing to the maintenance of neuronal functionality, neuronal development and distal axonal homeostasis (Ban et al., 2013; Fimia et al., 2007; Komatsu et al., 2007; Tang et al., 2014). Autophagic activity plays a role in the modulation of synaptic neurotransmission (Hernandez et al., 2012; Wang et al., 2015) and the maintenance of proper autophagic activity seems to be essential for neuronal functionality. The molecular players involved in the uniqueness of neuronal autophagy remain to be elucidated.

Is activation of neuronal autophagy a suitable approach to fight age-related diseases?

In order to fight neurodegenerative diseases, the search of compounds and small molecules with the ability to modulate the autophagic process has attracted a great deal of attention in the last years. Of note, the autophagosomal biogenesis does not seem to be impaired in most of the neurodegenerative disorders. Rather, accumulation of autophagosomes is found in the majority of neuropathologies (Nixon, 2013; Nixon et al., 2005) suggesting that autophagy malfunction might be due to deficits in fusion events with lysosomes or lack of acidification in the lumen of autophagic compartment. Therefore, the identification of novel therapeutic targets involved in late steps of autophagic maturation is now imperative.

Interestingly, the autophagic capacity declines with age and the gradual failure of autophagic function drives to the accumulation of damage in neurons (Cuervo, 2008). Genetic and pharmacological activation of autophagy has been proved to increase the lifespan of different model organisms and all these evidences point out the autophagy process as a druggable anti-aging mechanism (Gelino and Hansen, 2012). The development of novel autophagy modulators has been a hotspot in the field and different compounds have been identified holding the promise for extending the life span and for potential treatment of human diseases.

With no doubts, mTOR signaling represents the major route for autophagy modulation. Pharmacological inhibition of mTOR induces enhanced biogenesis of autophagosomes in multiple tissues and cellular types and it has emerged as a promising therapeutic target (Sarkar and Rubinsztein, 2008). However, it remains unclear if it is the case in neuronal autophagy because controversial *in vitro* an *in vivo* findings have been reported. In one hand, several reports have shown that the reduction of mTOR signaling with everolimus or rapamycin is not efficient to activate neuronal autophagy in cultured striatal or cortical neurons (Fox et al., 2010; Maday and Holzbaur, 2016; Tsvetkov et al., 2010). On the other hand, inhibition of mTOR signaling has been also shown to improve the clearance of neuro-pathological proteins through autophagy activation (Ravikumar et al., 2008; Ravikumar et al., 2004; Roscic et al., 2011). Of note, mTOR is a master regulator of autophagy by sensing the cellular energy viability. Nonetheless, neuronal autophagy

seems to be a deprivation-independent process suggesting that mTOR-independent pathways might be responsible for autophagy in neurons.

In addition to the inhibition of mTOR as therapeutic strategy, other mTOR-independent pathways modulate the autophagy initiation and they could be potential pharmacological alternatives (Williams et al., 2008). For example, the modulation of inositol pathway has been proposed as a potential druggable route thus low intracellular inositol triphosphate (IP3) levels would active autophagy. Examples of compounds with ability to influence the route of IP3 synthesis and enhance autophagy could be lithium or sodium valproate, already used in the treatment of epilepsy or bipolar disorders (Sarkar et al., 2009; Sarkar and Rubinsztein, 2008)

Intracellular concentration of Ca^{+2} is other factor modulating the autophagic process. A decrement of Ca^{+2} enhances autophagy activity and several FDA-approved Ca^{+2} channel antagonists and calcium channel blockers are considered potential therapeutic targets to modulate autophagy (Anekonda and Quinn, 2011; Williams et al., 2008).

Compounds with ability to modify the transcriptional levels of autophagy genes would be also potential candidates for modulating neuronal autophagy. Inhibitors of histone acetyltransferase activity or enhancers of deacetylase activity (i.e. spermidine or cilostazol, respectively) might induce pro-autophagic effects in neuronal autophagy (Eisenberg et al., 2009; Lee et al., 2015). In addition, a more efficient autophagic activity could be achieved by using chemicals with ability to adjust the activity of transcription factor EB (TFEB) that determine the expression of autophagic and lysosomal proteins essential for the autophagy process (Settembre and Ballabio, 2011; Settembre et al., 2011; Settembre and Medina, 2015).

Alternatively to drug development, the development of new technologies like induced pluripotent stem cells, new generation sequencing, safer and more efficient gene delivery mechanisms and the approval of gene editing for some pathologies in humans (as occurs recently for the CRISPR/Cas9 technology) make gene therapy a closer possibility for neurodegenerative diseases (Heman-Ackah et al., 2016; Yang et al., 2016). The genetic therapies could be aimed not only to reverse the mutations causing neurodegenerative diseases, but also to improve the activity of protective molecular pathways such as autophagy.

Quantitatively, the development of drugs has covered the major effort in the search of ways to upregulate the autophagic route, however, dietary manipulations could also be useful, cheaper, and safer alternatives to these pharmacological manipulations. For example, caloric restriction is able to activate autophagy contributing to extend the lifespan in different organisms (Bergamini et al., 2007). In addition, bioactive compounds present in dietary products might induce neuronal autophagy including members of the flavonoid family (i.e. resveratrol, curcumin or genistein), triterpenoids, isothiocyanates such as sulforaphane, vitamins (i.e. vitamin C, D3, K2) or specific types of fatty acids (Singletary and Milner, 2008). Of note, natural disaccharides such as trehalose has been already shown to enhance the removal of abnormal proteins in neurons through autophagic activity (Casarejos et al., 2014; Casarejos et al., 2011; Emanuele, 2014; Kruger et al., 2012; Perucho et al., 2012; Rodriguez-Navarro et al., 2010; Sarkar et al., 2007; Tanaka et al., 2004). Although promising, most of these studies lack of proper target engagement and

pharmacodynamics studies in humans. Therefore, further analysis will be required to establish the impact of these dietary constituents in neuronal autophagy. Finally, moderate physical exercise has been also proved to induce autophagy in multiple tissues including the brain (He et al., 2012). All these dietary strategies and moderate exercise may represent simple, non-toxic and low-cost manipulations to promote higher neuronal surveillance during aging or in neuro-pathologies.

Concluding Remarks

Drug development research conducted in the recent years revealed several therapeutic targets and compounds as autophagy modulators. For example, several FDA-approved drugs such as rapamycin or CCI-779 are shown to activate autophagy through the inhibition of mTOR signaling. However, it remains unclear if the mTOR inhibition is sufficient to modulate neuronal autophagy although other beneficial effects cannot be discarded in other cell types such as astrocytes, oligodendrocytes or microglia. In addition, since mTOR activity is involved in many other essential subcellular processes, these drugs are not considered ideal for long-term clinical use given that persistent inhibition of mTOR might lead to toxic side effects on patients.

Further work will be required to achieve a throughout understanding of neuronal autophagy revealing novel targets to modulate the autophagic route. Pharmacokinetics analyses in rodent brain to choose adequate doses for future clinical trials as well as careful examination of side effects will be required before considering these compounds as candidates for clinical therapies.

Above all, a better understanding of the role of autophagy in neurons and why, how and when a dysfunction of the process takes place during aging or in a specific neurological disorder is crucial for succeeding of any therapeutic approach. Accumulation of autophagosomes is described in most of neuropathologies and a hyperactivation of autophagosomal formation would not be beneficial, probably counterproductive. Future research shedding light in the late steps of the autophagic process will be required for a more efficient drug design.

In addition to pharmacological strategies targeting mTOR-dependent or mTOR-independent pathways, dietary manipulations, regular exercise or intermittent fasting could help to slow down the process of aging through the activation of autophagy.

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