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Review

Regulation and Roles of Autophagy at Synapses

Vassiliki Nikoletopoulou[1] and Nektarios Tavernarakis[1,2,*]

Genetic studies have demonstrated that conditional ablation of core autophagy genes in the neural lineage leads to progressive neurodegeneration, indicating that this catabolic pathway is indispensable for neuronal maintenance. However, accumulating evidence also indicates that autophagy is not merely a housekeeping process. Instead, autophagy may be dynamically regulated in different neuronal compartments and dictate the turnover of selected cargo in a time- and space-dependent manner and thus contribute to specialized neuronal functions. Here, we review the mechanisms regulating autophagy in neurons and discuss recent findings on the role of autophagy in synaptic morphology and function. Moreover, we discuss relevant questions that remain open and highlight forthcoming issues in the field of neuronal autophagy.

Highlights

Autophagy regulates neurotransmitter release across different species.

Autophagy is required for the developmental pruning of dendritic spines.

Autophagy is suppressed by BDNF signalling in the adult brain.

Modulation of autophagy may be required for different forms of synaptic plasticity.

Neuronal Autophagy: New Views

Autophagy is a catabolic process that degrades macromolecules and organelles by delivering them to the lysosome. Three mechanistically distinct types, microautophagy, chaperone-mediated autophagy, and macroautophagy, have been described [1,2]. Unlike the first two types, macroautophagy is a multistep process that entails the formation of a double-membrane vesicle, the autophagosome. The mechanisms of macroautophagy have been reviewed [3]. Briefly, autophagosome biogenesis starts with the assembly of a pre-autophagosomal structure (PAS), which is necessary for the **nucleation** (see Glossary) of a **phagophore**. The phagophore is a double-membrane structure that sequesters autophagic cargo as it elongates and closes to form the autophagosome. Here, we will focus on macroautophagy, which will be referred to hereafter as autophagy.

Autophagy was regarded as a housekeeping process that ensured the clearance of unwanted materials and prevented their aggregation in order to safeguard cellular function. Early work investigating the role of autophagy in the brain indicated that conditional ablation in the nervous system of core autophagy genes, such as *atg5* or *atg7,* led to the accumulation of aggregates of ubiquitinated proteins in neurons and late onset neurodegeneration [4,5]. However, recent work suggests that in addition to cellular maintenance, autophagy can also facilitate the homeostasis of specific proteins in a microenvironment or be regulated by neuronal signalling and activity, thus contributing to specialized neuronal functions. This is consistent with the realization that autophagy is not a generalized process but is capable of high selectivity in cargo sequestration. In the brain, several studies have delineated the requirement of autophagy in hypothalamic neurons for the regulation of nutrient uptake [6–9]. However, the role of autophagy in other neuronal functions, such as synaptic mechanisms that underlie complex cognitive functions, is only beginning to unravel. Here, we overview the regulation of autophagy in different neuronal compartments and discuss its roles in synapses.

[1]Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology Hellas, Heraklion 70013, Crete, Greece
[2]Department of Basic Sciences, Faculty of Medicine, University of Crete, Heraklion 71003, Crete, Greece

*Correspondence:
tavernarakis@imbb.forth.gr
(N. Tavernarakis).

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Autophagy at the Synapse

The synapse is a highly specialized neuronal compartment that forms the basic unit of communication between neurons. Communication relies on electrical signals that are propagated down the axon of the presynaptic neuron, where they trigger the quantal release of neurotransmitters into the synaptic cleft to elicit activity on the postsynaptic neuron. Both the presynaptic button and the postsynaptic density are specialized compartments, packed with molecules that are unique and dedicated to serving their function in neuronal communication. For example, presynaptic buttons are packed with synaptic vesicles containing neurotransmitters, as well as the machinery that allows fusion of these vesicles with the plasma membrane and the retrieval of fused vesicle membranes by endocytosis. On the other side of the synaptic cleft, highly specialized postsynaptic densities contain a high density of the receptors activated by the neurotransmitters, as well as scaffold, cytoskeletal, and signalling molecules, to ensure the activation of the postsynaptic neuron. In many neurons, including the pyramidal neurons of the neocortex, postsynaptic densities are mostly located on protrusions or outgrowths of dendritic shafts, known as **dendritic spines**, which increase the efficacy of neurotransmission [10]. The number of dendritic spines is developmentally regulated. In the mouse, synaptogenesis in the first 3 postnatal weeks is followed by a period of spine pruning that occurs between postnatal days 20 and 30 and is crucial for the proper wiring of the adult brain [11].

Anatomically, synapses are often located far away from the neuronal cell soma and it is intuitive to imagine that local mechanisms for safeguarding proper function may have developed. Emerging evidence, discussed below, suggests that autophagy is one homeostatic mechanism that has adapted to the microenvironment of the synapse in order to serve local functions related to synaptic transmission.

Autophagy in Presynaptic Buttons

In order to determine how autophagy is locally regulated in presynaptic buttons, the interaction of autophagosomes with proteins known to regulate synaptic vesicle trafficking has been investigated. Synaptojanin 1, a lipid phosphatase containing two distinct lipid phosphatase domains, 5-phosphatase and SAC1, each targeting different phosphoinositide phosphate (PtdInsP) species plays a role in this interaction. The 5-phosphatase domain has a preference for $PI(4,5)P_2$ as a substrate, while the SAC1 domain hydrolyses PI(3)P, PI(4)P, and $PI(3,5)P_2$. Synaptojanin 1 has been shown to hydrolyse $PI(4,5)P_2$ via its 5-phosphatase domain and lower the membrane-binding affinity of clathrin adaptors and uncoat newly formed vesicles [12].

In zebrafish cone photoreceptors, loss of synaptojanin 1 leads to specific accumulation of late endosomes and autophagosomes early in photoreceptor development, due to a defect in autophagosome maturation [13]. The 5-phosphatase domain was shown to be necessary for this process, but the SAC domain was dispensable [13]. By contrast, in *Drosophila* motor neurons, loss of synaptojanin 1 reduced the number of Atg8-positive puncta in synapses and prevented the accumulation of autophagosomes after blocking their fusion with the lysosome using chloroquine, suggesting that autophagosome biogenesis, and not maturation, is impaired [14]. Moreover, the SAC domain of synaptojanin 1, and not the 5-phosphatase domain, was found to be necessary for autophagosome biogenesis in axon terminals. Synaptojanin 1 mutants also caused the accumulation in synaptic buttons of structures positive for Atg18a, the *Drosophila* homolog of PROPPIN domain-containing proteins which recognize PI(3)P- and $PI(3,5)P_2$ to promote autophagosome biogenesis [15,16]. Therefore, the SAC1 domain may remove the $PI(3)P/PI(3,5)P_2$-binding protein, WIPI2/Atg18a, from immature autophagosomes, in a function that appears analogous to the hydrolysis of $PI(4,5)P_2$ by the 5-phosphatase domain.

Glossary

Dendritic spines: outgrowths on the dendrites of excitatory neurons, ranging in shape from mushroom, thorn-like, or filamentous, that are preferential sites of synaptic contact.
Long-term depression (LTD): the converse of long-term potentiation, a persistent decrease in synaptic strength. The long-lasting change in the postsynaptic neuron can be induced by a low-frequency electric stimulation of the presynaptic neuron.
Long-term potentiation (LTP): a persistent increase in synaptic strength. The long-lasting change in the postsynaptic neuron is in response to a transient input from the presynaptic neuron, such as a high-frequency stimulation. The persistence of this effect is demonstrated to extend many hours *in vitro* and several weeks *in vivo*.
NMDAR: It is an ion-channel receptor found at most excitatory synapses, where it responds to the neurotransmitter glutamate, and therefore belongs to the family of glutamate receptors. The NMDA receptor is one of three types of ionotropic glutamate receptors, the others being the AMPA and kainate receptors.
Nucleation: the first step in the formation of the phagophore via self-assembly of a pre-autophagosomal structure.
Omegasome: an omega-shaped structure on the membrane of the endoplasmic reticulum, where autophagosome biogenesis can occur.
Phagophore: the phagophore, also known as the 'isolation membrane', is a double-membrane structure that sequesters autophagic cargo and will eventually close to form the autophagosome.

Interestingly, an R258Q mutation in synaptojanin 1 is pathogenic for Parkinson's disease (PD), and was shown to impair autophagy and lead to the loss of dopaminergic neurons, both in *Drosophila* and in humans [14]. Notably, several other proteins that are implicated in PD pathogenesis were recently shown to regulate presynaptic autophagy. For example, the LRRK2 kinase, the most commonly mutated protein in PD, also affects autophagy within synaptic terminals [17]. In the case of LRRK2, this appears to occur via phosphorylation of the essential presynaptic membrane factor EndophilinA at serine 75 [17–19], which regulates synaptojanin 1 activity [12]. A phospho-mimetic of EndophilinA (EndoS75D) or an active LRRK2 (G2019S) were both shown to increase Atg8-positive puncta in presynaptic buttons and promote the colocalization of Atg3 with Atg8 [17,20]. Therefore, EndophilinA is proposed to create docking sites for Atg3 on expanding autophagosomes and was recently reported to cause neurodegeneration in mice [21]. Transcriptional and proteomic analyses of endophilin-A mutant mice revealed an upregulation of the E3-ubiquitin ligase FBXO32/atrogin-1, which, like EndophilinA, tabulates membranes and localizes to clathrin-coated structures and its transcription factor FOXO3A. Additionally, this work suggested that both FBXO32 and endophilinA are necessary for autophagosome formation, and both colocalize transiently with autophagosomes [21].

However, the role of autophagy in presynaptic release of dopamine has been directly investigated, by conditionally ablating atg7, a key component of the autophagic machinery, in dopaminergic neurons [22]. Chronic autophagy deficiency resulted in increased size of axonal profiles, increased evoked dopamine release, and rapid presynaptic recovery. The inhibition of mammalian target of rapamycin (mTOR) with rapamycin also acutely increased the number of autophagosomes in axons, decreased the number of synaptic vesicles, and attenuated the release of dopamine in wild type animals [22].

Recent work has suggested that autophagy may regulate neurotransmitter release through an interaction between Atg16L, a protein present in phagophores, and Rab26, which is present on the membrane of synaptic vesicles [23]. Rab26 is a less-studied member of the Rab superfamily, and it was previously identified by proteomic analyses to be present on synaptic vesicles [24,25]. Rab26 preferentially localizes to large clusters of synaptic vesicles and tends to oligomerize in its GDP-bound form. The core autophagy protein Atg16L1 is an effector of Rab26 that binds to the GTPase exclusively in the GTP-form. Although Atg16L1 has been described to perform noncanonical functions not related to autophagosome formation [26], it is likely that Rab26-Atg16L1 complexes in neurons represent PAS, as LC3 is recruited to these complexes.

Clustering of synaptic vesicles is proposed to occur at times of rest, in order to reduce the mobility of individual synaptic vesicles [27]. Moreover, it has been reported that synaptic vesicle clusters are capable of redistributing to nearby active sites. Previously, several endocytic proteins have been shown to localize to these clusters, such as intersectin 1/Dap160 [28,29] and dephosphins [30,31], however, the functional significance of this localization is not clear. In addition to the localization of Atg16L1 to synaptic vesicle clusters, several autophagy-independent functions have been proposed for Atg16L1. For example, Atg16l1 is shown to localize on hormone-containing dense core vesicles of neuroendocrine cells, where it interacts with Rab33A and facilitates their release [32]. Therefore, while it is very likely that the Rab26-Atg16L1 interaction facilitates the recruitment of the autophagic machinery onto synaptic vesicles clustered by Rab26 to facilitate their clearance, as a means of homeostatically regulating the number of synaptic vesicles available for release, there could be alternative scenarios. For example, Atg16l1 may merely be a component of the proteinaceous matrix that holds synaptic vesicles together in a

cluster or helps with their migration to nearby active sites. To help discern these possibilities, it would be necessary to further characterize how presynaptic autophagy in general and the Rab26-Atg16L1 interaction itself are affected by neuronal activity.

Regulation of presynaptic autophagy by signalling pathways known to play roles in neurotransmitter release remain to be investigated. For example, the Wnt signalling pathway is a known regulator of synaptogenesis, which also regulates synaptic vesicle clustering and neurotransmitter release [33]. Autophagy in cancer cells can be inhibited by Wnt signalling [34]; conversely, autophagy can inhibit Wnt signalling by degrading proteins such as Dishevelled that are required for signal transduction [35].

In addition, brain derived neurotrophic factor (BDNF), as a key mediator of activity-dependent modifications of synaptic strength, is known to increase the number of synaptic vesicles docking at active sites and enhance quantal neurotransmitter release [36]. In light of our findings that BDNF supresses autophagy in neurons (Figure 1), it is conceivable that BDNF may specifically supress presynaptic autophagy. In support of this, analysis of conditional BDNF deletion in the nervous system shows an overabundance of autophagosomes primarily in presynaptic compartments, as determined by electron microscopy analysis in the hippocampus [37].

Recent work has also demonstrated that the presynaptic protein Bassoon, which is crucial for the release of synaptic vesicles [38], acts to actively inhibit autophagy in the presynaptic compartment [39]. Loss of Bassoon in cultured hippocampal neurons results in increased numbers of autophagosomes, as determined by electron microscopy. Bassoon is proposed to inhibit autophagy by binding to Atg5, an E3-like ligase that is crucial for the attachment of LC3 to autophagosomes. In the absence of Bassoon, increased presynaptic autophagy is accompanied by decreased numbers of synaptic vesicles, also suggesting that autophagy actively degrades synaptic vesicles or their components.

In addition to degrading synaptic vesicles, presynaptic autophagy could also have other cargoes. In a mouse model of tuberous sclerosis complex 2 (Tsc2), known to result in overactivation of mTOR signalling and reduced autophagy, there was an accumulation of mitochondria in the presynaptic compartment [40]. Using a microfluidic device to separate axonal compartments, damaging mitochondria triggered mitophagy in distal axons by a mechanism that requires PINK1 and Parkin [41]. Given the central role of mitochondria in neurotransmitter release [42], it is possible that mitophagy is actively occurring near the active zone to ensure the elimination of defective mitochondria. Development of novel genetic tools that will allow us to monitor endogenous mitophagy, similar to those developed in *Caenorhabditis elegans* [43], will be valuable in order to observe mitophagy in mammalian neurons under physiological conditions and further characterize its role in different neuronal compartments, including the presynaptic sites.

Taken together, several lines of evidence suggest that autophagy directly regulates neurotransmitter release, by mechanisms that remain to be fully elucidated. Moreover, presynaptic proteins also regulate autophagy locally to adjust cellular degradation to the changing demands of the presynaptic compartment. Presynaptic autophagy has recently been discussed in a dedicated review [44].

Autophagy in Postsynaptic Sites

Much less is known about the signals and mechanisms regulating autophagy on the postsynaptic site, and especially in dendritic spines that facilitate most excitatory neurotransmission.

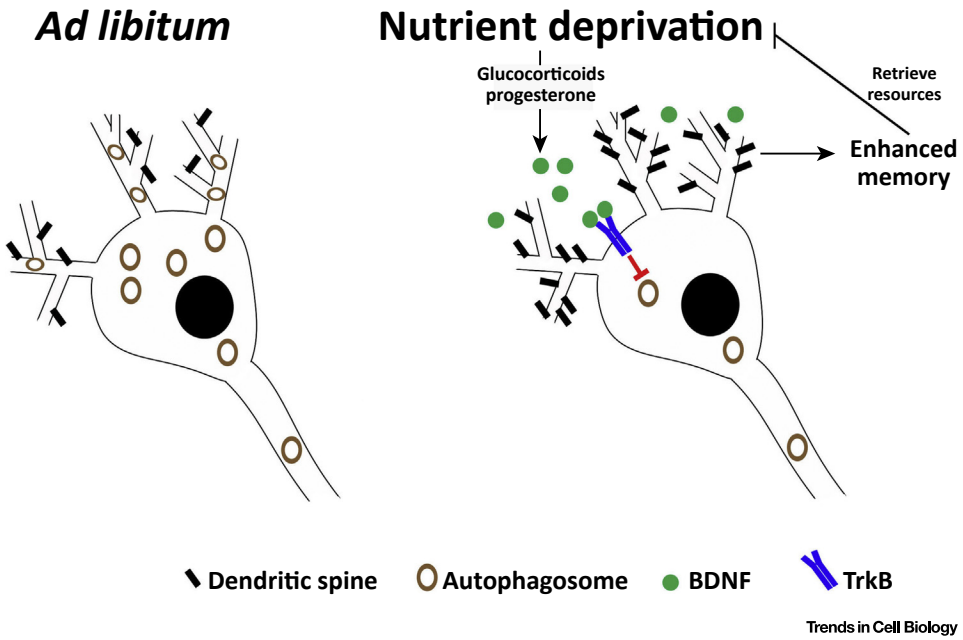
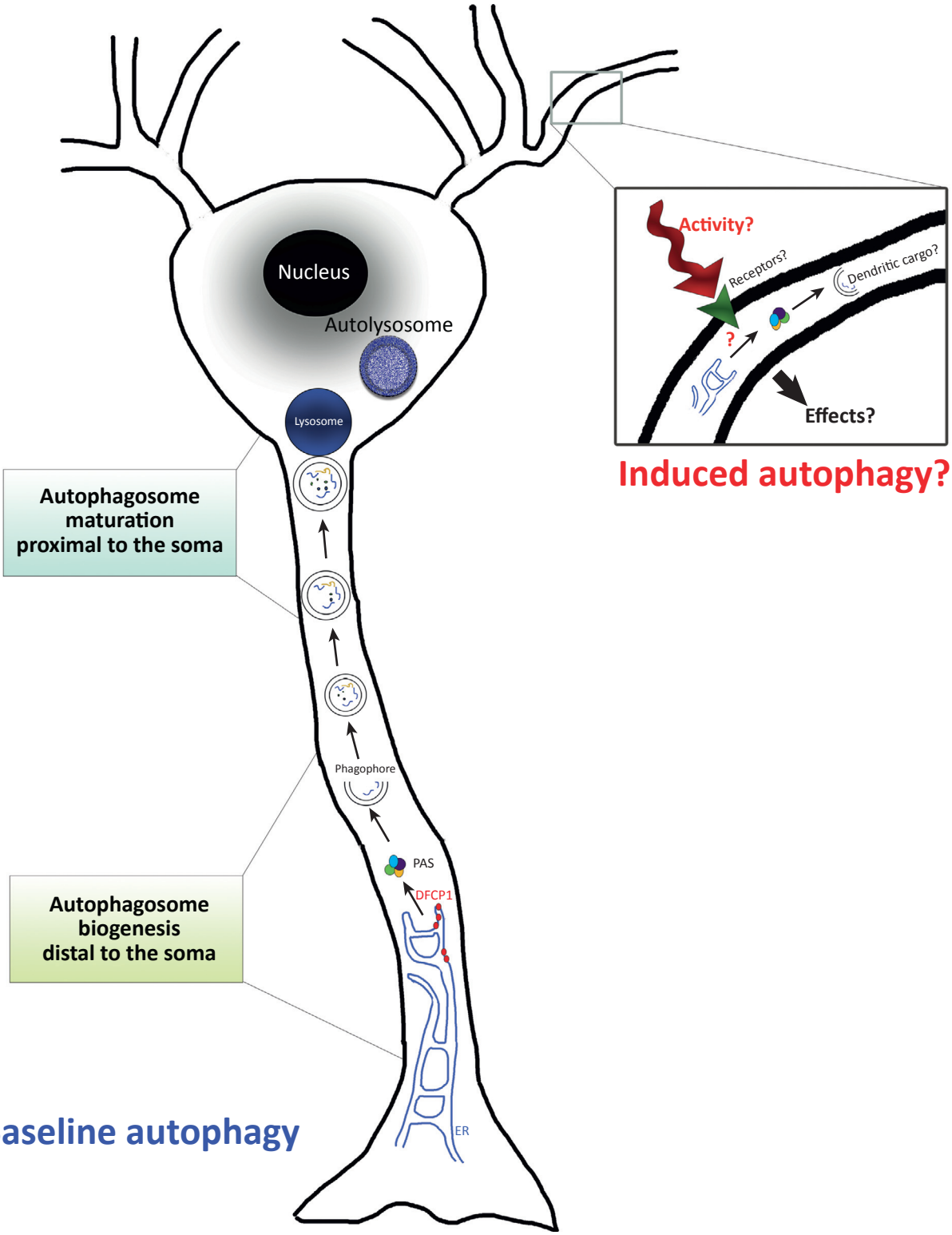


Figure 1. Schematic Representation of the Regulation of Neuronal Autophagy by BDNF Signalling. Nutrient deprivation causes the upregulation of BDNF in the forebrain, which is mediated by glucocorticoids. Increased BDNF signalling via the TrkB receptor suppresses autophagy by inhibiting autophagosome biogenesis. This leads to an increased number of dendritic spines and enhanced memory, which could be an adaptive response that enhances fitness and facilitates food retrieval. Abbreviations: BDNF, brain derived neurotrophic factor; TrkB, tyrosine kinase receptor B.

The first evidence indicating that autophagy may regulate postsynaptic organization came from experiments on *C. elegans*, while investigating the effects of de-innervation on the clustering of Gamma-aminobutyric acid A ($GABA_A$) receptors on the postsynaptic membrane of muscles [45]. Consistent with previous studies in *C. elegans* [46], this work demonstrated that GABA terminals provide a signal to the postsynaptic cell to initiate synaptic $GABA_A$ receptor clustering and trafficking [45]. Moreover, it revealed that in the absence of presynaptic innervation, autophagy is upregulated in the postsynaptic cell, and $GABA_A$ receptors accumulate in autophagosomes and are targeted for degradation. These findings, for the first time, demonstrated that regulation of autophagy at the synapse facilitates the crosstalk between the pre- and postsynaptic sides. However, the mechanisms and signalling pathways by which the postsynaptic cell regulates autophagy locally in response to presynaptic innervation remains elusive. Moreover, as autophagosomes were also detected in dendrites, it is unknown if autophagic vesicles can be formed locally in dendrites or whether they are transported from the axon tip (Figure 2). A discussion on autophagosome biogenesis can be found in Box 1.

In addition, mTOR-dependent autophagy was recently found to be necessary for developmental spine pruning [47], as conditional deletion of *atg7* in excitatory neurons resulted in aberrant pruning and an increased number of spines. These morphological defects were accompanied by autistic-like behaviours, consistent with previous findings indicating that patients with autism-spectrum disorders exhibit dendritic spine dysgenesis and often increased number of spines [48]. A discussion on the role of autophagy in cognitive function and its impairment can be found in Box 2.



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Box 1. Autophagosome Biogenesis in Neurons

Autophagosome biogenesis follows a highly ordered and conserved sequence of events that requires the participation of numerous autophagy proteins and membrane trafficking components [99]. This process starts with the formation of a phagophore, a cup-shaped double-membrane structure, which is believed to form *de novo* by nucleation from a pre-existing membrane. The membrane source of the phagophore is debated, and could possibly depend on the cell type, the signalling pathway triggering autophagy, or the confinement of the process in microenvironments that will lead to the sequestration of specific cargo. As autophagy is often studied under starvation, it is, to a large extent, agreed that under these conditions phagophore nucleation occurs on **omegasomes**, omega-shaped structures near endoplasmic reticulum (ER)-mitochondria contact sites labelled by the protein DFCP1. Other described membrane sources include other ER sites, such as the ER-exit sites or ER-Golgi intermediate compartments (ERGIC), the Golgi, recycling endosomes, or the plasma membrane [99].

As neurons are uniquely intricate in morphological diversity and complexity, two questions emerge: first, whether phagophore nucleation occurs throughout the neuron, and second, what are the membrane sources for the phagophore in neurons? In peripheral neurons of transgenic mice expressing GFP-LC3, autophagosome biogenesis was found to occur in axon tips [100]. As cultured peripheral neurons are not synaptically connected, the study was extended to include synaptically active cultured hippocampal neurons, which found that the same principle of autophagosome biogenesis in distal axons also applied [101].

The functional importance of this spatial restriction has not been experimentally addressed. Moreover, as the ER is widely distributed in neurons, [102], it would be interesting to elucidate what makes the ER of the axon tip uniquely capable of donating membranes to the phagophore. It would also be worth examining whether autophagosome biogenesis can occur more freely at different locations (such as dendrites or axon hillock) under conditions that induce autophagy above the baseline (Figure 2).

Using fluorescently tagged Atg13 and Atg5 proteins, it was shown that Atg13 and Atg5 are recruited concomitantly onto nascent autophagosomes, followed by DFCP1. This is an unexpected finding, considering that Atg5 is recruited downstream of both Atg13 and DFCP1 [103] and it raises the question of whether the large fluorophore tags of these proteins could alter their properties. The source of the phagophore membrane was also investigated and it was concluded that the phagophore nucleates from DFCP1-positive foci on the ER [101]. Autophagosome biogenesis in the presynaptic compartment was shown to involve specialized proteins, as discussed 'Autophagy in Presynaptic Buttons'.

As developmental spine pruning is mediated by mechanisms similar to those facilitating the shrinkage and elimination of dendritic spines during persistent depression of synaptic strength [49], the question arises whether autophagy is regulated during **long-term depression (LTD)** and whether it contributes to the structural changes associated with LTD expression. In cultured neurons, brief low-dose NMDA treatment, known to confer chemical LTD, caused autophagy upregulation [50]. This work also suggested that autophagy may function to locally degrade internalized AMPA receptors. However, it remains unclear whether autophagy upregulation has a physiological role in mediating LTD and whether it contributes to receptor degradation. It is also possible that autophagy may degrade scaffold proteins, which are responsible for keeping receptors anchored to the postsynaptic membrane, or cytoskeletal proteins that maintain dendritic spine structures. Moreover, signalling proteins or lipids that facilitate neurotransmission downstream of receptor activation are also putative autophagic cargo, as their abundance would directly affect neurotransmission. These may include diacylglycerols, key messengers that modulate certain PKC isoforms, or inositol-1,4,5-trisphosphate (IP3) which releases Ca^{2+} from intracellular stores, both known to play a role in excitatory neurotransmission. Novel approaches allowing the detailed characterization of the cargo of purified neuronal autophagosomes by unbiased proteomic and lipidomic analyses will shed light onto the diversity of synaptic cargo that may be relevant to synaptic function.

Figure 2. Schematic Representation of Autophagosome Biogenesis in Neurons. Autophagosome biogenesis under baseline conditions was shown to occur preferentially in axon tips, distal to the soma, from DFCP1+ foci on the ER. By contrast, autophagosome maturation is described as taking place proximal to the soma. Abbreviations: DFCP1, double FYVE-containing protein 1; ER, endoplasmic reticulum; PAS, pre-autophagosomal structure.

Box 2. Autophagy in Cognitive Function and Dysfunction

It is widely accepted that synaptic plasticity is the cellular mechanism underlying the complex behaviours that comprise cognition. For example, memory and learning are encoded by **long-term potentiation** of synaptic strength, while long-term depression is implicated in responses that require behavioural flexibility. Consistently, disorders such as autism spectrum disorders (ASD) and schizophrenia, which entail synapse dysgenesis and are collectively referred to as synaptopathies, are characterized by severe cognitive and intellectual deficits [104].

Notably, many syndromic forms of ASD are caused by mutations in proteins associated with mTOR signalling, such as PTEN, TSC1, TSC2, and FMRP. Previous work has focused on the dysregulation of protein translation via the deletion of these genes, however recent studies have examined how disruption of autophagy, another major downstream branch of mTOR signalling, contributes to neuronal dysfunction and ASD pathogenesis. To this end, it was revealed that mice with conditional deletion of *Atg7* by CaMKII-cre, which are deficient for autophagy in forebrain excitatory neurons, exhibit ASD-like behavioural deficits and an increased number of spines [47]. In addition, conditional ablation of autophagy in microglia also impairs synaptic pruning and causes social behavioural deficits associated with ASD [105], highlighting a requirement for autophagy in the immune system for proper synaptic function. Moreover, *de novo* mutations in activity-dependent neuroprotective protein (ADNP) were found in a syndromic form of ASD, and ADNP was recently found to enhance neuronal autophagy [106]. Consistent with these findings, small exonic copy number variations from whole exome sequence data of patients have recently highlighted dysregulation of autophagy in ASD [107]. Autophagy has also been recently implicated in the pathogenesis of schizophrenia, based on reduced levels of ADNP and Beclin1 in postmortem tissues of patients [108]. Notably, these findings have led to phase II clinical trials for schizophrenia utilizing NAP (davunetide), a peptide fragment of ADNP which enhances the ADNP-LC3 interaction [109].

Regulation of Neuronal Autophagy

The TOR Pathway

The TOR signalling pathway is known to play a key role across species at the interface of signals that determine the balance between cell growth and degradation in response to changes in nutritional status, growth factor availability, and stressors. In various mammalian cell types, activation of mTOR efficiently suppresses autophagy, mainly by phosphorylating components of the PAS, such as ULK1 and Atg13 [51], which inhibits their assembly and prevents autophagosome biogenesis [52–54]. However, the role of mTOR in regulating autophagy in neurons has been controversial and is discussed below.

Tsc1 is a critical negative regulator of mTOR, hence, its ablation leads to mTOR overactivation. The analysis of brains from mice with conditional ablation of *Tsc1* in pyramidal neurons clearly demonstrated that *Tsc1* mutants exhibit reduced autophagy in pyramidal neurons [47]. Furthermore, administration of rapamycin prevents this, thus, targeting mTOR overactivation as the cause of the reduced autophagy. However, in the same study, the effects of systemic rapamycin administration on autophagy levels in control animals appears to be marginal, suggesting that while mTOR overactivation can dramatically supress autophagy, inhibition of endogenous mTOR is not sufficient to robustly increase baseline autophagy in the brain. This observation is in line with some *in vitro* studies demonstrating that inhibition of mTOR by rapamycin or Torin1 is not sufficient to induce autophagy in cultured cortical or hippocampal neurons [55,56]. By contrast, in cultures of dopaminergic neurons, isolated from the ventral midbrain, and in *ex vivo* striatal slices, rapamycin was shown to effectively increase the number of autophagosomes in the cell bodies and neurites [22]. Therefore, it remains possible that the requirement of mTOR in regulating baseline autophagy may differ among distinct types of neurons. However, this hypothesis has not yet been tested *in vivo*, by examining autophagy induction in different brain areas and neuronal subpopulations of wild type mice after systemic administration of rapamycin.

Starvation

Starvation represents the best and most widely studied paradigm of autophagy induction. It is well established that autophagy is rapidly induced in many tissues upon nutrient deprivation, by

mechanisms that are mainly mediated by suppression of the aforementioned TOR signalling pathway [57]. Several studies have demonstrated that autophagy seems to be readily induced by starvation in cultured forebrain [58,59] and hypothalamic neurons *in vitro* [7]. In addition, deprivation of glucose was found to attenuate the retrograde transport of autophagosomes within 4 hours [55]. Moreover, sex differences have been reported, indicating that starvation induces a more profound increase in autophagic flux in male cultured neurons [60].

Despite the consistent *in vitro* findings, the effect of starvation *in vivo* is controversial. Early work using GFP-LC3 transgenic mice showed that while a 24-hour starvation caused the clear conversion of LC3-I to the lipidated and autophagosome-associated LC3-II species in most tissues, this was not the case in the brain [57]. These biochemical findings were supported by morphological analyses, which indicated that while the size of the liver was reduced upon starvation, the size of the brain remained unaffected [57]. Therefore, it is possible that to safeguard organismal fitness, other organs will undergo autophagic degradation to provide the brain with nutrients, with the brain being the last to suffer nutritional stress.

More recently, the same GFP-LC3 transgenic autophagy reporter mice combined with imaging of autophagosomes, showed a profound induction of autophagy in the cortex and in Purkinje cells in the cerebellum after 24 or 48 hours of starvation [61]. LC3 signal was detected in the cortex, however, it was not possible to resolve cellular identity as neuronal subtype was not determined. It is worth noting that Purkinje cells are GABAergic interneurons, which form the minority of neurons in the cerebellum. Likewise, the cortex also contains a minority of GABAergic interneurons that comprise approximately 30% of the total population. It has not yet been resolved *in vivo* at the neuronal subtype level whether starvation can induce autophagy in interneurons, but not in pyramidal cells, thus explaining the discrepancies between biochemical and imaging studies. More recently, starvation was also found to induce autophagy in the hypothalamus [7].

By monitoring endogenous LC3 and p62 levels, recent work from our labs demonstrated that autophagy is differentially regulated by starvation in different brain regions [37]. In the murine adult brain, starvation caused an upregulation of autophagy in the hypothalamus, but an unexpected suppression in the cortex and hippocampus. Although cell type specificity was not addressed in this study, the cellular composition of these brain areas is very different, with the forebrain containing a majority of glutamatergic pyramidal neurons, while the hypothalamus contains the highest diversity of neurons in the brain and only about 30% of these neurons are glutamatergic [62]. However, starvation caused a widespread induction of autophagy both in the forebrain and the hypothalamus, in newborn and juvenile animals until postnatal day 40 [37]. These results suggest that, as neural networks in the brain mature well into early adulthood, neuronal autophagy is differentially regulated not only spatially but also temporally. For most of the murine forebrain, postnatal day 40 corresponds to adolescence, a period soon after the end of developmental spine pruning, which is a major event in the shaping of neural networks.

One major consideration is that the brain, unlike the liver, is not a homogeneous tissue. In addition to the presence of non-neuronal cells, such as astrocytes, oligodendrocytes, and microglia, neurons themselves exhibit enormous subtype diversity and they are organized in networks and spatially distinct structures that are dedicated to different functions [63,64]. Therefore, future work should aim at investigating the induction of autophagy by starvation at the neuronal subtype level.

Neurotrophin Signalling

BDNF is the major growth factor for the central nervous system (CNS), where it is required for neuronal maturation and long-term synaptic plasticity (LTP) [65–71], among other functions. In rat hippocampal neurons cultured for 5 days, withdrawal of the commonly used B27 supplement mimics starvation and causes an upregulation of autophagy and neuronal death [72]. Using this paradigm, application of BDNF suppressed the autophagic flux in an mTOR-dependent manner and rescued neuronal death. Moreover, addition of BDNF to mature cultured cortical or hippocampal neurons caused a significant suppression of the autophagic flux, which was mediated by activation of its cognate receptor tyrosine kinase receptor B (TrkB) and downstream activation of the PI3K/Akt pathway [37]. In this paradigm of mature cultured neurons, rapamycin could only partially rescue the suppression of autophagy, indicating that regulation of mTOR signalling downstream of BDNF [73] is not the only mechanism mediating the suppression of autophagy.

Consistent with the *in vitro* observations, conditional ablation of BDNF in the nervous system caused an increase in LC3-II levels and overabundance of autophagosomes, as determined by electron microscopy [37]. Interestingly, impairment of LTP caused by BDNF deficiency could be fully rescued by pharmacological inhibition of autophagy, suggesting that increased neuronal autophagy can impair LTP and memory formation. This is in line with the observations that stimuli that induce LTP activate mTOR [74] and that inhibition of mTOR attenuates LTP and memory formation [75]; however these findings have been considered only in the context of impaired protein synthesis and not in the light of autophagy modulation.

In addition, it was reported that activation of the cognate BDNF receptor TrkB causes its localization to autophagosomes. This work proposed that autophagosomes are responsible for the retrograde transport of activated TrkB [76], mediated by an interaction of LC3 with the endocytic adaptor AP2. Therefore, it is likely that there is negative feedback regulation by the autophagic machinery to terminate BDNF signalling by delivering activated TrkB to the lysosome for degradation.

Although BDNF is not required for the survival of neurons in the CNS [77], altered levels have been associated with several neurodegenerative disorders. Whether the regulation of autophagy by BDNF is relevant in neurodegeneration has not been directly examined. There are consistent findings for a role of BDNF is in Huntington's disease (HD), especially because the trophic role of anterogradely transported cortical BDNF on medial spiny neurons of the striatum, which are amongst the most vulnerable neurons in HD, is well characterized [77]. Production of cortical BDNF is under the stimulatory control of huntingtin (htt) but is lost in mutant htt, leading to decreased levels of BDNF in the cortex [78,79]. Interestingly, mutant htt also contributes to the activation of autophagy as determined in cell, rodent, and human patient tissues, by sequestering and inactivating mTOR [80]. However, the mechanisms of cargo recognition appear to be impaired in HD [81]. Given the suppression of autophagy by BDNF signalling, it is therefore possible that increased autophagy in HD may be partially mediated by decreased BDNF signalling.

In addition, there is some evidence that the neurotrophin receptor $p75^{NTR}$ can induce neuronal autophagy in an *in vitro* model of cerebellar death [82]. More specifically, withdrawal of serum from cultured Purkinje neurons causes a caspase-independent programmed cell death which is distinct from apoptosis and is characterized by excessive autophagosome-lysosomal vacuolization. Treatment with the prototypic neurotrophic factor nerve growth factor (NGF) can rescue this form of death by suppressing autophagy via p75NTR; in the absence of NGF unligated p75 is responsible for the induction of autophagy and the ensuing cell death. The *in vivo* relevance of p75 signalling in the modulation of neuronal autophagy is not yet determined.

Neuronal Activity

Neuronal activity mediates basic synaptic transmission and forms the basis for long- and short-term changes in synaptic function. Interestingly, it was only recently demonstrated that neuronal autophagy can be regulated by neuronal activity [50]. Using *in vitro* neuronal cultures, a brief, low-dose pulse of *N*-methyl-d-aspartate (NMDA), which is known to chemically induce LTD of synaptic strength, rapidly and robustly increased the autophagic flux and the number of GFP-LC3 puncta in dendrites and in spines, by activating the NMDA receptor, **NMDAR** [50]. NMDA is an amino acid derivative that mimics the function of the excitatory neurotransmitter glutamate and activates the glutamate NMDA receptor (NMDAR), but not other ionotropic glutamate receptors, such as kainate or AMPA receptors. Systemic administration of kainic acid, a specific agonist of kainate receptors, causes a transient increase in LC3-II levels in the hippocampus 4–6 hours later [83]. However, it remains unclear whether, in addition to NMDAR, activation of other ionotropic glutamate receptors, such as AMPA or kainate receptors, can modulate neuronal autophagy and whether this has a physiological role.

Downstream of NMDAR activation, the induction of autophagy was shown to depend on protein phosphatase 1 and PTEN and on the inhibition of mTOR [50]. LTD is a persistent form of plasticity that underlies many cognitive functions and is expressed by shrinkage or elimination of dendritic spines on excitatory dendrites. As the brief low dose of NMDA used in this study is known to induce a chemical form of LTD that mimics LTD *in vivo*, these findings raise the question as to whether induction of autophagy is required for LTD or for other forms of synaptic plasticity and, if that is the case, what is the specific cargo that autophagy degrades to contribute to structural changes at the synapse?

Moreover, in addition to NMDAR-LTD, a second major form of LTD in CNS synapses is the one mediated by activation of group I metabotropic glutamate receptors (mGluRs) [84]. Although both NMDAR- and mGluR-LTD both result in shrinkage and elimination of dendritic spines, they are mechanistically distinct and they are known to coexist in many CNS synapses [84]. It remains unknown whether activation of these mGluRs and downstream g-protein coupled signalling can also activate autophagy and whether this is necessary for the ensuing structural changes.

Secretory Autophagy in Neurons?

In addition to degradation, autophagy may also constitute an unconventional secretory pathway, facilitating the secretion of proteins such as interleukin-1b or galectins that lack signal peptides [85] and has been reviewed elsewhere [86]. Briefly, secretory autophagy was shown to depend on Atg5 [85,87] and is believed to require most other proteins that participate in the nucleation and elongation of autophagosomes. The parting between the degradative and secretory routes likely depends on trafficking molecules that direct autophagosomes either to the lysosome or alternatively to the plasma membrane. For example, while the small GTPase Rab8a, a regulator of polarized sorting to the plasma membrane, was shown to be needed for secretory autophagy [85,88], its closely related isoform Rab8b is more important for degradative autophagy [89]. More recently, it has been suggested that targeting of autophagosomes to the plasma membrane depends on the interaction of Sec22b, a SNARE protein anchored on the outer membrane of secretory autophagosomes, with syntaxin 3/4 as well as SNAP-23/29 on the plasma membrane [90].

Neurotransmission and communication between neurons and other cell types requires the controlled release of molecules from synaptic vesicles and large dense-core vesicles. It remains unclear whether secretory autophagy represents an additional secretory route in neurons, which is employed under physiological and/or pathological conditions (Figure 3). Recent

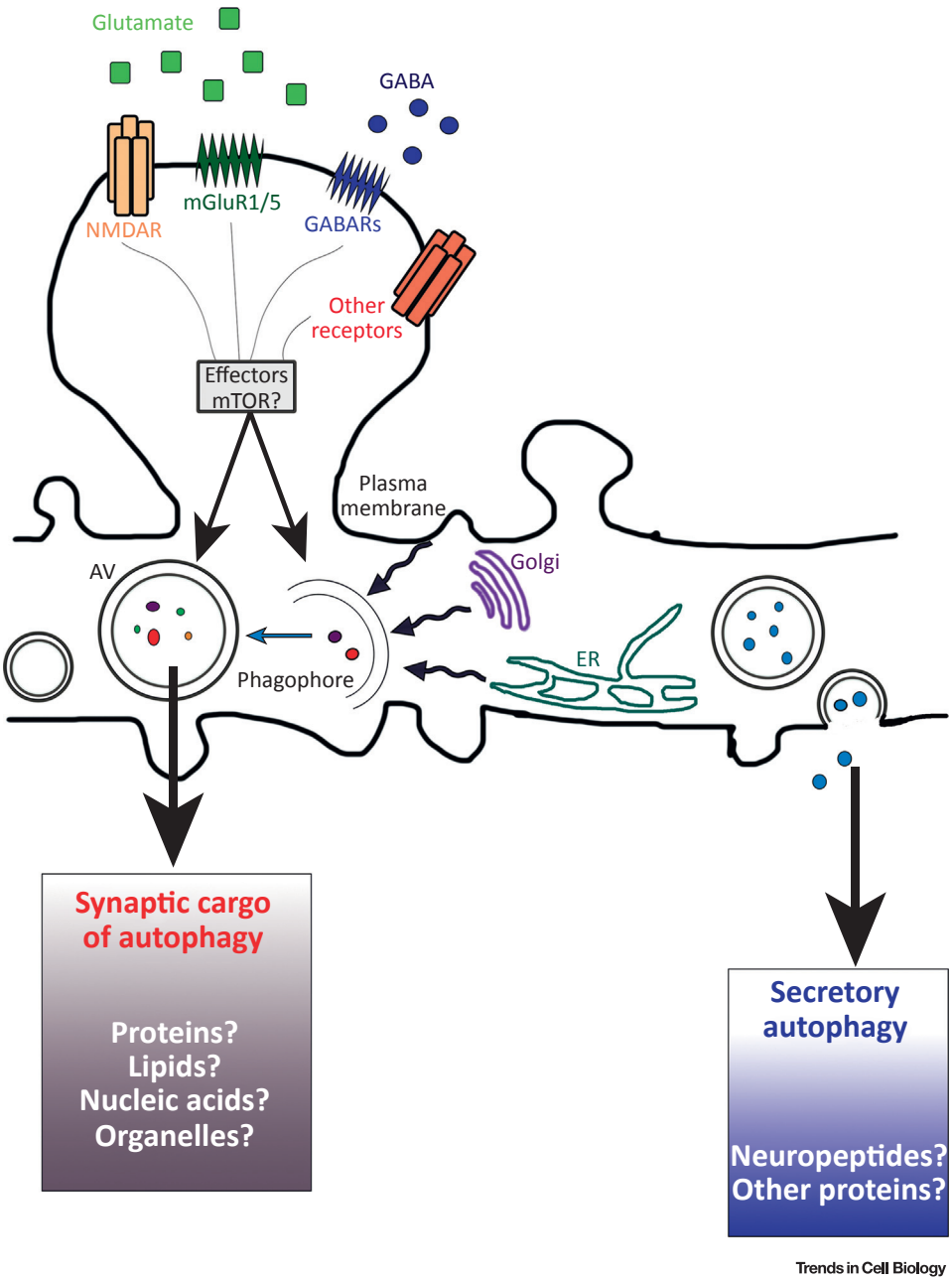

Figure 3. Schematic Representation of Autophagy Induction in Dendrites. Different neurotransmitters, such as glutamate or GABA, may modulate neuronal autophagy in dendrites by activating receptors located on postsynaptic densities via mechanisms that may involve the mTOR pathway. It remains unclear if, upon neuronal activity, phagophores can be formed locally in dendrites, or if they are transported there from axonal compartments. The source of the phagophore membrane could potentially originate from diverse compartments, such as the plasma membrane, the Golgi, or the ER, as indicated by wavy arrows. Moreover, the dendritic cargo of autophagy is also elusive, as is any potential regulation of secretion via autophagy. Abbreviations: AV, autophagic vesicle; GABA, Gamma-aminobutyric acid; GABARs, GABA receptors; mGluR1/5, metabotropic glutamate receptor 1/5; mTOR, mechanistic target of rapamycin; NMDAR, *N*-methyl-D-aspartate receptor.

studies have suggested that secretion of mutant huntingtin, cytosolic amyloids, and a-synuclein may rely on mechanisms dependent on prelysosomal compartments, the molecular nature of which has not yet been determined [91–93].

In addition, secretory autophagy has also been implicated as an unconventional mechanism for the insertion of proteins into the plasma membrane. One such example is Mpl, the receptor for thrombopoietin, which plays a crucial role in megakaryocytic differentiation and maturation. While the fully glycosylated, mature Mpl receptor follows the canonical secretory pathway, the core-glycosylated, immature protein is inserted into the plasma membrane by secretory autophagy [94]. In addition, hundreds of neuronal surface membrane proteins, which include a substantial fraction of surface $GABA_A$ receptor or AMPA-type and NMDA-type glutamate receptor subunits, are core-glycosylated, thus exhibiting unconventional secretory processing [95]. This results in the neuronal membrane and synaptic sites displaying high levels of glycosylation profiles that are classically associated with immature intracellular proteins [95]. Whether the trafficking of these core-glycosylated receptors to the postsynaptic density or other neuronal sites requires some autophagic intermediate is a very exciting possibility that remains to be addressed. Exploring the regulation of secretory autophagy in neurons and the nature of its cargo can add new dimensions in neuronal communication and in delineating the roles of autophagy in neuronal function and dysfunction.

Concluding Remarks and Future Directions

Convergent evidence supports a model whereby neuronal cells have developed novel and highly specific mechanisms for regulating autophagy. These mechanisms potentially allow neurons to uniquely and unconventionally regulate this crucial catabolic pathway in space- and time-dependent manners.

The physiological significance of autophagy in neuronal function is steadily emerging. The studies discussed herein reveal that key neuronal functions, such as neurotransmitter release, pruning of dendritic spines, and behavioural outputs of neural networks are all shown to depend to some extent on local regulation of autophagy. Future work should concentrate on working out the details, as autophagy is likely to have adapted different functions in different neuronal subpopulations and in different forms of plasticity. As protein turnover is known to be crucial for synaptic plasticity, the contribution of autophagy to these processes should be more directly examined, as well as its interaction with the proteasome, which is known to be modulated by neuronal activity and plasticity (see Outstanding Questions) [96–98]. As an initial step towards understanding how the homeostasis of proteins, signalling lipids, nucleic acids, and organelles are dictated by autophagy, novel methods are needed to characterize the diversity and dynamic nature of the autophagic cargo during different neuronal processes. These include the isolation and purification of autophagosomes from different neuronal compartments to gain insight into cargo species, the distinction of secretory from degradative autophagosomes, and finally, the development of genetically encoded reporters for monitoring selective autophagy, such as mitophagy, in mammalian neurons, *in vitro* and *in vivo*.

Outstanding Questions

Is modulation of autophagy required for the induction of LTD or the structural synaptic changes necessary for its expression?

Most studies have focused on the role of autophagy either in glutamatergic or in dopaminergic synapses. Does autophagy have a role in shaping inhibitory synapses in the brain?

What is the synaptic cargo of autophagy? Is it dynamically regulated by neuronal activity?

Under baseline conditions, autophagosome biogenesis was shown to be restricted to distal axons. How is this restriction achieved and what purpose does it serve? Can autophagosome biogenesis occur at other neuronal compartments upon neuronal activity or synaptic plasticity?

Under baseline conditions, DFCP1-positive foci on the endoplasmic reticulum were shown to be the sole membrane donors for the phagophore in neurons. Can the pre- or postsynaptic plasma membrane or other organelles donate membranes to phagophores upon neuronal stimulation?

Is secretory autophagy occurring in neurons? In that case, what is the nature of neuronal proteins that are secreted by autophagy? Is secretory autophagy important for neuronal plasticity or synaptic function?

A crosstalk between autophagy and the ubiquitin-proteasome system (UPS) has been described in non-neuronal cellular contexts. How does autophagy interact with the UPS in neurons? Do they have distinct and/or coordinated roles in synaptic function?

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