

Review Article

Keeping synapses in shape: degradation pathways in the healthy and aging brain

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Synapses maintain their molecular composition, plasticity and function through the concerted action of protein synthesis and removal. The complex and polarized neuronal architecture poses specific challenges to the logistics of protein and organelle turnover since protein synthesis and degradation mainly happen in the cell soma. In addition, post-mitotic neurons accumulate damage over a lifetime, challenging neuronal degradative pathways and making them particularly susceptible to the effects of aging. This review will summarize the current knowledge on neuronal protein turnover mechanisms with a particular focus on the presynapse, including the proteasome, autophagy and the endolysosomal route and their roles in regulating presynaptic proteostasis and function. In addition, the author will discuss how physiological brain aging, which entails a progressive decline in cognitive functions, affects synapses and the degradative machinery.

Introduction

Protein homeostasis, or proteostasis, refers to all protein synthesis and degradation processes that preserve the essential balance of cellular protein levels. Synapses are especially dependent on the mechanisms for maintaining proteostasis as they contain an extremely dense network of proteins that needs to undergo constant remodeling to govern neurotransmission and synapse reshaping during learning and memory processes [1,2]. Most synapses are located far away from the soma, the main compartment for protein synthesis and degradation, creating extra challenges to the logistics of proteostasis pathways. In addition, because of their post-mitotic and long-lived nature, neurons and their synapses are at increased risk for age-related damage accumulation. During aging, cells encounter stressful situations, caused for instance by a buildup of unfolded proteins or an accumulation of reactive oxygen species (ROS) causing lipid peroxidation and protein oxidation [3]. At the synapse, high metabolic burdens and calcium insults during neuronal activity might even accelerate protein damages and turnover [4,5]. The burden on mature and aging synapses increases even more, as aging is associated with a progressive decline in the efficiency of degradation systems, such as the proteasome, endolysosomal and autophagy pathway. This review will first summarize current knowledge on the proteostasis processes that act to remove dysfunctional synaptic components (Figure 1) and how, for instance, these pathways are locally regulated to maintain proper functioning of the presynapse. Second, this review will address how physiological brain aging affects the synapse and its degradative capacity.

Ubiquitin–proteasome system (UPS)

A central feature of all major degradative pathways is the ability to selectively target substrates by site-specific mono- and poly-ubiquitination. Ubiquitin is a 76-amino acid protein that covalently attaches to substrate proteins. This conjugation relies on the sequential activities of three classes of enzymes: E1 (ubiquitin-activating), E2 (ubiquitin-conjugating) and E3 (ubiquitin ligases). Ubiquitination is initiated by E1, an enzyme requiring ATP for the activation of ubiquitin. Ubiquitin is then handed over to an

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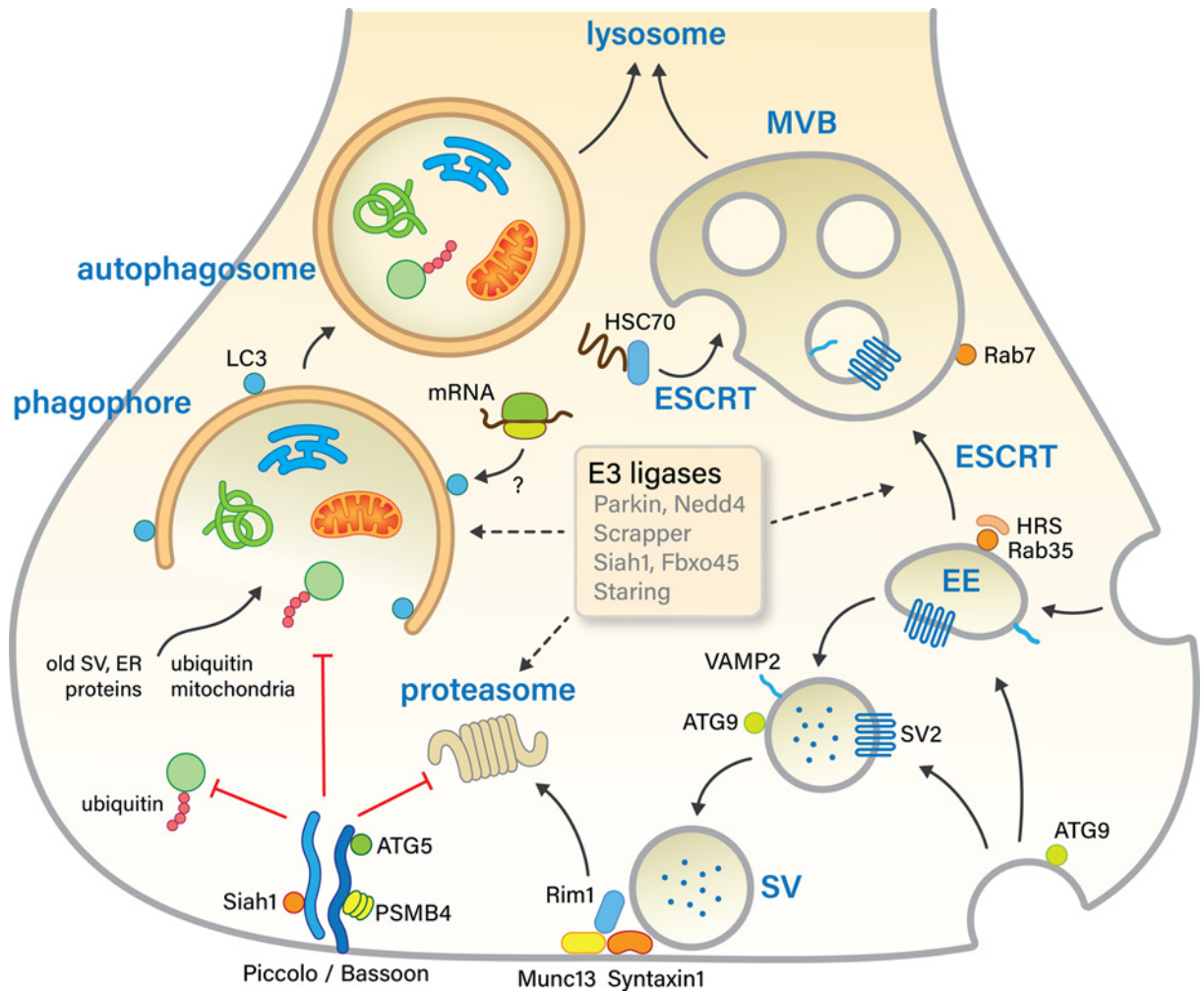


Figure 1. Degradative pathways and suggested presynaptic cargos

Synaptic proteins and organelles, such as SVs and mitochondria, can be locally removed via alternative degradative routes. The proteasome removes predominantly cytosolic proteins, including active zone proteins RIM1 and Munc13. ESCRT-dependent endolysosomal sorting is thought to be required for the degradation of a subset of membrane proteins, for instance, SV proteins VAMP2 and SV2. Both proteins and organelles can be sequestered by a double-membrane structure called a phagophore, which then closes to form an autophagosome that eventually fuses with somatic lysosomes for degradation. Ubiquitination is a common signal for degradation in all of these major protein degradation pathways. Therefore, ubiquitin-ligases (E3) and deubiquitinating enzymes could play important roles in maintaining a healthy presynapse. The six E3 ligases indicated here either have known, mammalian, presynaptic targets (Nedd4, Scrapper, Fbxo45, Staring) or are locally regulated by presynaptic proteins (Siah1, Parkin); EE, early endosome; SV, synaptic vesicle; MVB, multivesicular body.

E2 to form an ubiquitin~E2 conjugate via a thioester exchange reaction. The (>600 known) E3 ligases mediate the final step of ubiquitin transfer and provide the specificity for substrate recognition by interacting with both E2 and the target protein [6]. Ubiquitin can create different types of poly-ubiquitin chains via its lysine residues and each type of chain has the potential to act as a distinct intracellular signal. For instance, K48-linked chains are a prime signal for the ubiquitin–proteasome system (UPS) whereas K63-linked chains play a more prominent role in lysosomal degradation [7]. Deubiquitination enzymes (DUBs) serve as an important regulatory layer and contribute to the replenishment of ubiquitin pools.

The highly conserved UPS is responsible for the degradation of the majority of short-lived, cytosolic proteins. The proteasome consists of a barrel-shaped 20S catalytic core particle and 19S regulatory particles that select and unfold target proteins labeled with ubiquitin chains. During presynaptic assembly, a local decrease in UPS activity is proposed to aid in the formation of a stable cluster of synaptic vesicles (SVs) [8–10]. Research over the years also demonstrate

critical functions for the UPS in mature synapses, with UPS involvement in the regulation of basal neurotransmission and synaptic plasticity [2,11,12]. Synaptic plasticity includes long-term potentiation (LTP) and long-term depression (LTD) that are thought to form the cellular basis of learning and memory, and their dysfunction may underlie memory loss and other cognitive disorders [13,14]. Incubation of hippocampal slices with proteasome inhibitors enhances early phase LTP induction [15] while impairing late phase LTP maintenance [11,16]. Bilateral infusion of lactacystin, a specific proteasome inhibitor, in the hippocampal CA1 area of rats is shown to impair memory formation and memory extinction [17,18]. The mechanisms that underlie proteasome-dependent plasticity have not been fully elucidated but probably depend on changes in the postsynaptic compartment [17,19]. In addition, proteasome inhibition is implicated in presynaptic neurotransmitter release. At the *Drosophila* neuromuscular synapse, acute pharmacological inhibition of the proteasome in the presynaptic neuron results in stabilization of active zone protein Unc-13/Munc13 and increased synaptic transmission due to a more efficient priming and release of SVs [20]. In cultured rat hippocampal neurons, proteasome inhibition causes an increase in the size of the recycling pool, although in this study no effect on SV release was observed [21]. Proteasome 20S complexes are mainly present in the cytosol, but a recent finding indicates that they can associate with the plasma membrane of neurons, where they are involved in the activity-dependent degradation of intracellular proteins, producing extracellular peptides that can modulate neuronal activity [22]. It is currently unclear how these surface-exposed proteasomes target to the plasma membrane and are embedded into the lipid bilayer.

Apart from changes in neurotransmission, proteasome dysregulation in mature synapses also causes structural presynaptic abnormalities. Inactivation of PI31, an adaptor for microtubule-based transport of proteasomes, results in the accumulation of ubiquitinated protein aggregates in mouse and fly axon terminals and is accompanied by distal axon swellings and presynapse loss [23,24], while leaving the postsynapse mostly unperturbed [23]. Whereas in these studies the identity of the accumulated proteins was not determined, other works have indicated that the levels of some presynaptic proteins, such as synaptophysin, liprin-alpha, RIM, Bassoon and above-mentioned Munc13 are controlled via UPS-mediated degradation [25–28]. At the mature mammalian presynapse, ubiquitin E3 ligases have been identified to induce proteasomal degradation of presynaptic proteins (Figure 1). The E3 enzyme Staring, for instance, ubiquitinates syntaxin 1, a member of the SNARE complex and an essential component of the SV fusion machinery [29]. E3 ligases SCRAPPER and Fbxo45 ubiquitinate active zone proteins RIM1 and Munc13, thereby contributing to the UPS-dependent regulation of SV release. Accordingly, loss of these E3 ligases results in a facilitation of neurotransmission [30,31]. In addition, the presynaptic active zone (AZ) proteins Bassoon and Piccolo are known to bind and inhibit the E3 ligase Siah1 [32]. Bassoon has also been found to interact with PSMB4, a structural subunit of the 20S core proteasome, thereby interfering with compartment-specific ubiquitination and proteasome biogenesis [33]. Accordingly, loss of Bassoon and Piccolo increases ubiquitination and degradation of SV proteins, eventually resulting in SV loss and synapse elimination. Thus, these AZ proteins seem to stabilize the AZ by inhibiting degradation. Interestingly, core AZ components show age-induced increases in *Drosophila* brains [34], but whether mammalian neurons show similar trends is unknown. Regarding the role of synaptic deubiquitylation very little is known. At the postsynapse, USP46 is identified as the DUB for AMPARs, stabilizing cell-surface expression levels [35]. Our knowledge regarding presynaptic ubiquitin regulation, including the identity, roles and substrates of DUBs is still very limited and thus warrants further investigation.

While the UPS locally and preferentially targets short-lived soluble proteins, the endolysosomal and autophagy pathways are responsible for the elimination of membrane proteins, long-lived proteins, insoluble protein aggregates and dysfunctional organelles [36]. In neuronal axons and synapses, both the endolysosomal and autophagy pathways are retrograde trafficking routes that deliver proteins to degradative lysosomes that are primarily present in neuronal somata [37].

The endolysosomal pathway

Transmembrane proteins and associated factors are typically degraded by the endolysosomal system and access this pathway by endocytosis. Pre-existing early endosomes then fuse with these endocytosed vesicles, permitting proteins to recycle back to the cell surface (regulated by small GTPases Rab4 and Rab11) or directing cargo to late endosomes or late endosomal multivesicular body (MVB) and finally to lysosomes for degradation (regulated by Rab7) [38]. During their transport towards the soma, axonal endosomes gradually mature and acidify which may also control their degradative capacity [37,39]. In the pathway promoting degradation, Endosomal Sorting Complexes Required for Transport (ESCRT) is a core protein machinery, important for the recognition of ubiquitinated membrane cargo and the formation, trafficking, and fusion of MVBs. The ESCRT machinery is composed of multiprotein complexes known as ESCRT-0, I, II and III. The ESCRT-0 protein HRS initiates the process by binding to ubiquitinated cargos and tethering them to the surface of early endosomes by binding phosphatidylinositol 3-phosphate (PI[3]P). On

endosomes, PI[3]P is generated by the Vps34 phosphatidylinositol 3-kinase complex II (Vps34/Vps15/Beclin1) that assembles at early endosomes via the association of Vps15 with Rab5 [40]. Recruitment of Rab5 effectors, such as Rabaptin-5 and Rabex-5, can regulate Rab5 activity and the formation of a local endosomal PI3P pool [41]. The sequential recruitment and activation of the other ESCRT complexes promotes inward vesicle budding and membrane sealing by the Vps4 ATPase [42]. Studies on ESCRT-dependent presynaptic protein degradation are scarce but evidence from *Drosophila* and cultured rat hippocampal neurons suggests that endosomal sorting is required for the degradation of a subset of ubiquitinated SV proteins (e.g. VAMP2 and SV2), a process involving active Rab35 dependent HRS recruitment to SV pools [43,44]. In agreement with this, mutations in CHMP2B, an ESCRT-III subunit involved in frontotemporal dementia, results in an increase in presynaptic endosomes and a selective retention of SV-associated proteins in aged mice [45]. In contrast, at the *Drosophila* photoreceptor axon terminals, sorting of SV proteins was found to be independent from Rab7 whereas degradative sorting of plasma membrane proteins was Rab7-dependent, indicating the involvement of multiple degradative sorting pathways [46]. Cytosolic proteins can also enter the endolysosomal system by “endosomal microautophagy”, a pathway dependent on ESCRTs, but not the core autophagy machinery (see below). This process relies on the cytosolic chaperone Hsc70 for substrate targeting and the subsequent invagination-mediated uptake by late endosomes/MVBs. In *Drosophila* neurons, endosomal microautophagy mediates the turnover of synaptic proteins harboring a KFERQ recognition motif, including for instance Unc-13/Munc13 and EndophilinA, thereby rejuvenating the protein pool and facilitating synaptic transmission [47]. Given the fact that in the presynapse most organelles and markers for the early stages of the endolysosomal route are present, including MVBs, Rabs and ESCRT components [48–50], this route might play an underappreciated but important role in synaptic proteostasis and function and therefore represent an interesting area for future research.

Autophagy at the presynapse

Although often used synonymously, there are at least three distinct types of autophagy; microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy. Microautophagy (similar to endosomal microautophagy) is defined by the direct uptake of cargo by the lysosome via lysosomal membrane invagination [51]. In CMA, selective cytosolic substrate proteins bind the chaperone Hsc70 and are thought to be translocated across the lysosomal membrane via the receptor lysosome-associated membrane protein type 2A (LAMP2A) [52]. The most prevalent form of autophagy, however, is macroautophagy (hereafter called autophagy) that utilizes double membrane vesicles, called autophagosomes, that deliver their content to the lysosome. Under baseline conditions, neuronal autophagosomes are mostly generated in the axon tip and retrogradely transported to the cell soma for fusion with degradative lysosomes [53]. Autophagosome formation requires a “core machinery” that starts with initiation factor ULK1 kinase complex, which translocates to the endoplasmic reticulum (ER) membrane (or another membrane donor) to activate the VPS34/ATG14/Beclin1 complex, that in turn triggers the production of PI[3]P. The subsequent recruitment of the PI[3]P effector WIPI2B allows for further recruitment of autophagy-specific ubiquitin-like conjugation systems, including E1-like ATG7 and the E3-like ATG12-ATG5:ATG16L ligase complex, that lipidate LC3 and LC3-related proteins. The lipidated form of LC3 stably inserts itself into the growing inner and outer autophagosome membrane to promote membrane elongation, substrate recruitment and finally autophagosome closure [54]. Autophagy can be either a non-selective bulk process, or a selective process that utilizes so-called autophagy receptors that can recognize substrates and simultaneously interact with lipidated LC3, targeting specific substrates to the inner membrane of the growing autophagosome. The specific action of these receptors is regulated by protein modifications, such as ubiquitination, phosphorylation and oligomerization [55]. Autophagosomes are then transported to the soma in a dynein-dependent manner, a process that is linked to their maturation and gradual acidification, for example by fusion with late endosomes or lysosomes to form autolysosomes [56]. Interestingly, the canonical machinery involved in synaptic transmission can also act to locally regulate presynaptic autophagy. Presynaptic endocytic proteins Endophilin and Synaptojanin, for instance, promote autophagosome formation by aiding the lipidation of LC3 and autophagosome closure while the endocytic adaptor complex AP2 facilitates retrograde axonal transport of mature autophagosomes (also referred to as amphisomes) to promote TrkB/BDNF signaling [57]. Conversely, the AZ protein Bassoon inhibits autophagy by binding to autophagy protein ATG5 [58–61].

Autophagosome biogenesis and transport in axons have been studied in detail [62], and emerging studies now tackle the question what substrates are targeted by neuronal autophagy and how this process is involved in maintaining synapse function. At the post-synapse, recent studies show that dendritic autophagy is required for synaptic LTD and the removal of postsynaptic AMPA receptor subunits and scaffold proteins [63,64]. The main sites of autophagosome biogenesis are at distal axons where autophagosomes are proposed to contain cargoes such as damaged SV proteins [65,66] but also mitochondria [67,68], ER [69] and whole SVs [67,70]. Similar to proteins,

organelles have finite lifetimes and accumulate damages over time that can, if not taken care of, lead to organelle dysfunction. In addition, during endo-exocytotic cycles, SVs are gradually contaminated with proteins that normally reside on the plasma membrane. According to one hypothesis the accumulation of a certain number of these proteins prevents aged SVs from fusion with the PM. Hence, such aged SVs need to be degraded [71]. In mouse dopaminergic neurons, induction of autophagy by treatment with the mTOR inhibitor rapamycin results in an ATG7-dependent reduction in the number of SVs and a consecutive decrease in evoked neurotransmitter release [72], suggesting that SVs as a whole can be targeted to the autophagy pathway. The synaptic vesicle-associated GTPase Rab26 is postulated to connect SV recycling and autophagy by binding to autophagy protein ATG16L and directing SVs as a whole to pre-autophagosomal structures [70,73]. *Rab26* null mutant fruit flies suffer from defective stimulus-dependent neurotransmission, although this did not appear to be accompanied by changes in autophagosomal and SV markers, suggesting a possible autophagy-independent function of Rab26 [74]. Recent work indicates a role for ATG9, the only known transmembrane protein involved in the core autophagy pathway, by linking the activity-dependent SV cycle with autophagosome formation. ATG9 exhibits lipid scramblase activity and, together with the lipid transport protein ATG2, mediates the flow of lipids from a donor membrane to the growing autophagosome membrane [75]. ATG9 is enriched at presynaptic terminals where it plays a role in local autophagosome biogenesis [76]. Recent work in *Caenorhabditis elegans* and mouse primary neurons shows that ATG9-containing vesicles undergo exo-endocytosis at presynaptic sites in an activity-dependent manner, via shared components of the machinery for SV recycling. Mutants that disrupt synaptic endocytic traffic, including a Parkinson's disease (PD) associated mutation in synaptojanin1/*unc-26*, result in presynaptic ATG9 accumulation and accelerate the age-dependent decay of *C. elegans* synapses and locomotor behavior [77]. How ATG9 cycling aids in autophagosome biogenesis and what autophagy substrates underlie the reported age-dependent changes remains to be explored.

Modulation of presynaptic neurotransmission by autophagy is not only proposed to occur via removal of SVs and their associated proteins. Knockout of the core autophagy protein ATG5 selectively in excitatory forebrain neurons results in increased excitatory neurotransmission at CA3-CA1 synapses in acute mouse hippocampal slices and in primary hippocampal cultures, an effect independent from SV number or SV protein levels [69]. Instead, the gain in neurotransmission results from elevated calcium release from endoplasmic reticulum (ER) tubules, which accumulates at presynaptic sites as a consequence of abolished autophagy. Another independent study also failed to detect major alterations in the levels of SV proteins in brains lacking ATG5 specifically in either excitatory or inhibitory neurons. Instead, they identified autophagy-dependent degradation of synaptic subunits of protein kinase A (PKA), a key regulatory enzyme involved in the phosphorylation of the synaptic proteome [78]. In contrast, a recent study using proteomic analysis of isolated autophagic vesicles from wild-type mouse brain tissue suggested synaptic proteins as basal autophagy cargoes [67]. This may reflect differences between the analysis of cargoes under wild-type conditions or cargo accumulation in autophagy inhibited condition, a situation in which other mechanisms might be able to take over degradation of certain components. The same study revealed mitochondrial fragments as a major cargo within neuronal autophagosomes. Due to their role in sequestering cytosolic calcium or ATP production, presynaptic mitochondria can regulate presynaptic release, plasticity and increase synapse stability [79,80], suggesting that autophagy-mediated removal of axonal mitochondria modulates presynaptic processes. Indeed, in aged rodents and fruit flies, mitochondria lose their functionality possible as a consequence of malfunctioning autophagy [81,82] (and see further below). Future studies will be needed to determine how synaptic cargo is recognized and what autophagy receptors and protein modifications might aid substrate recognition.

To allow neuronal synapses to modify their proteome during processes like plasticity, regulated protein degradation, but also protein synthesis must be implemented (for a nice overview on synaptic protein synthesis and delivery see [83]). Traditionally believed to be a purely somatic event, synthesis of proteins from messenger RNA (mRNA) can occur locally at the distal presynapse [84]. Interestingly, a recent study shows that the autophagy protein LC3B is an RNA-binding protein that can trigger rapid mRNA degradation upon autophagic activation in HEK293T cells, thereby mediating cross-talk between autophagy and local protein synthesis [85]. It would be interesting to see if LC3-dependent RNA degradation plays a role in regulating the local synaptic proteome.

Common signals and regulators of degradative pathways

Degradative pathways can respond to a multitude of stressors, including protein damage and nutrient starvation. These signaling pathways can regulate degradation pathways in different ways. For example, inhibition of the mechanistic target of rapamycin (mTOR) signaling is responsible for autophagy induction during nutrient starvation and neuronal activity [86–88] but is also involved in proteasomal inhibition [89]. Interestingly, mTOR is present at

synapses. Here, its inactivation decreases the strength of evoked excitatory synaptic transmission via both post- and presynaptic mechanisms, including enhanced spontaneous SV vesicle fusion [90]. Another signal that can influence both UPS and autophagy is proteostatic imbalance in the ER (known as ER stress). ER stress can be a result of abnormalities in calcium homeostasis, oxidative stress and other conditions leading to protein folding defects, processes postulated to play an important role in the rate of aging. To restore balance, ER stress activates many different pathways, including autophagy and the unfolded protein response (UPR), a stress response mediated by ER transmembrane stress sensors (PERK, ATF6 and IRE1 α) that induces transcriptional and translational programs to lower protein production and promote degradation. ER stress and the UPR are commonly associated with neurodegenerative diseases [91], and UPR manipulations can restore cognition and synapse function in aged mice [92,93]. Recent studies suggest that UPR can be locally initiated by activity in the distal dendrite and at postsynapses, thereby inducing dendritic growth [94], but the exact contributions of this broad process to synapse maintenance are presently unknown.

Although the above discussed degradation pathways use different molecular machineries, some of the key players are shared between them. A common denominator of all is the selection of substrates by ubiquitination. Ubiquitin ligases as well as DUBs can be shared between the degradation pathways. The E3 ubiquitin ligase PRKN/Parkin for instance, can catalyze a range of ubiquitination events, including mono-ubiquitination, K48- and K63-linked poly-ubiquitination. These events can mark substrates for the proteasome [95] but also play an important role in autophagic degradation of mitochondria [96] and, as more recently suggested, presynaptic vesicle autophagy [97]. Another E3 ligase, named Nedd4, induces rapid mGlu7 degradation via both proteasomal and lysosomal pathways [98]. Similarly, ubiquitin receptors can convey selectivity but are sometimes involved in multiple pathways, for instance, the autophagy receptor p62 brings ubiquitinated proteins to the autophagosome via LC3 binding but is also involved in proteasomal degradation [99]. As indicated above, the large AZ protein Bassoon can modulate the presynaptic proteome in several ways, by negatively regulating autophagy [60], proteasome activity [33] and protein ubiquitination [32]. Components of the degradative pathways are themselves often also substrates, proteasomes for example, can be degraded by bulk autophagy [100]. In addition, the MVB and autophagy pathways merge at the late endosome/lysosome [101], making both pathways sensitive to a block in lysosomal degradation by, for instance, proton pump or phosphoinositide 3-kinase (PI3K) inhibitors. Thus, when targeting specific protein degradation pathways or proteins, effects on downstream machineries or redundancy of degradative pathways need to be taken into consideration.

Global synapse changes during normal aging

Even in the absence of neurodegenerative diseases, the functional capabilities of our brain, such as memory and processing speed, progressively decline with age. Synapses form the requisite connections of the neuronal networks that underlie cognition and therefore represent an important target for the effects of aging. Human synapse formation starts prenatally and lasts until about 2 years after birth, after which half of all synapses are subsequently “pruned” during the following two decades [102]. After this developmental period, a proportion of synaptic connections will be stably maintained over life spans of months to years [103]. Other connections, however, are unstable with turnover rates in the range of a few days to weeks, likely depending on the brain region [104–106]. Alterations in synaptic turnover are correlated with the ability to learn and to retain information, indicating the importance for neuronal circuit plasticity [107,108]. A growing body of evidence supports the idea that disruptions in these synaptic connections drive brain changes and cognitive decline that occur during normal aging [109]. Decreases in spine density have been reported in aging rodents [92,110–112] (however also see [113,114]), non-human primates [115], and humans [116]. *In vivo* two-photon imaging of cortical spines and boutons in old mice (>20 months) revealed changes in basal synapse dynamics but not number [116,117] with for instance a reduction in learning-induced new spine formation [111]. The hippocampus, an area essential for learning and episodic memory, is particularly sensitive to aging [118] and a reduction in SV numbers is observed in aged hippocampal mossy fiber (MF)-CA3, and CA3-CA1 synapses [119]. These structural synaptic changes observed during aging also lead to functional and behavioral deteriorations. Electrophysiological studies in the context of aging consistently show changes in synaptic transmission, excitability and plasticity. In general, aging is often associated with a decreased synaptic transmission and a reduced ability to increase synaptic strength through LTP [120,121]. Not all synapses, however, are equally affected by aging. For instance, aged rodents display defective presynaptic LTP at MF-CA3 synapses [119] and reduced excitability in CA1 [122] but hyperactivation in CA3 pyramidal neurons [123]. A recent study combining electron microscopy and behavioral experiments, set out to link hippocampal CA1 and CA3 synaptic architecture with cognitive ability during aging. They demonstrated that synapse architecture did not change in old rats that were performing as well in

learning-tasks as their young counterparts. In contrast, old but learning-impaired animals exhibited structural synaptic changes only in CA3 synapses. Notably, synapses within hippocampal region CA3 showed an increased number of perforated synapses, a synapse characterized by gaps in post- and presynaptic densities, and a different distribution of synaptic weight as measured by AMPAR expression [124]. Thus, although in some brain regions specific neuronal loss is observed during aging processes [125], it is clearly the more subtle changes in plastic synaptic connections that drive cognitive decline during normal aging. Other studies set out to address what molecular changes occur that could underlie these age-induced changes in synaptic connections. Studies have shown age-related decline of synaptic proteins such as synaptophysin [126] and NMDA receptor subunits [127]. Recently, multiple large datasets have become available that include age-related neuron and synapse changes in mice, monkeys and humans on multiple levels including the transcriptome [128–133], proteome [92,134–136], lipidome [137], epigenome [138] and metabolome [139]. These studies now provide an important basis for identifying signatures for cell changes during aging; however, we still lack detailed understanding on what underlies these changes and how they can be directly related to synaptic connectivity changes and cognitive decline during aging.

In agreement with previous studies in *C. elegans* [140,141], a recent study employing *in vivo* metabolic labeling and proteomic profiling of mouse brains, correlated normal aging of wild-type mice with decreases in global protein recycling [134]. Why global proteostasis declines during normal aging processes is not well understood but includes decreases in the efficiency and cooperation of protein quality control systems. The next paragraphs will focus on how physiological aging affects the major presynaptic protein degradation routes.

Age-related changes in presynaptic degradation pathways

During normal brain aging, malfunctioning of autophagy processes are described in many organisms such as *D. melanogaster*, rodents, and humans. This includes changes in mTOR activity, gene expression, down-regulation of autophagy proteins (including ATG5, ATG7 and Beclin), and decreases in the amount of autophagosomes or autophagic flux [142–146]. A number of studies have shown that autophagy contributes to memory formation [63,147,148] and emerging studies in mice demonstrate a link between age-induced impairment of hippocampus-dependent memories and the down-regulation of certain autophagy factors, highlighting the need for functional neuronal autophagy in learning and memory. Importantly, genetic manipulations restoring autophagic function are sufficient to rescue age-related memory decline [147]. Similarly, the autophagy inducer spermidine protects from age-related synaptic alterations at murine hippocampal MF-CA3 synapses and counteracts age-dependent memory impairment in mice and fruit flies [82,119,149]. Moreover, elevated dietary spermidine intake is associated with a lower risk for cognitive impairment and decline in humans [82]. How autophagy is differentially modulated at aging presynapses remains unclear. Age-induced increases in the levels of the active zone scaffolding protein Bassoon might inhibit autophagy and, thereby, reduce release probability and synaptic transmission. Studies performed in primary mouse dorsal root ganglion neurons have revealed an age-dependent decrease in autophagosome biogenesis, caused by a local loss of the PI[3]P effector WIPI2B. This leads to the stalling of autophagosomes and failure to recruit LC3 at axon terminals. Strikingly, overexpression of WIPI2 was sufficient to return axonal autophagosome biogenesis to levels observed in younger neurons [150]. Whether hippocampal synapses experience similar age-associated autophagy impairments remains to be determined.

The relatively newly discovered chaperone-mediated autophagy (CMA) also decreases with age, predominantly due to changes in lysosomal lipid composition leading to reduced stability of LAMP2A [151]. Recent work on rodent models with neuron-specific LAMP2A loss demonstrated changes reminiscent of brain aging, including accumulation of oxidized and ubiquitinated proteins, synapse loss and memory impairments [52,152]. Although presynaptic accumulation of α -synuclein is observed after CMA loss [153], there has been little research on CMA at synapses and its role in modulating synaptic content and function needs to be determined.

Defects in the neuronal endosome-lysosome and ESCRT pathway, including pH increases and enlargement of early endosomes, are one of the earliest observed changes in neurodegenerative diseases such as Alzheimer's disease (AD) and PD [154]. However, few data are available on changes in this pathway during normal brain aging. Recent findings identified the BAG3-HSP70 complex as a mediator of endolysosomal degradation in rat cortical neurons, exerting its effects by disinhibiting Rab35 activity [155]. Interestingly, BAG3 expression is decreased in AD brain pathology [156] but is increased during normal aging [157]. Whereas ESCRT proteins control the pathway towards lysosomal degradation, another protein complex called Retromer (i.e. comprising the proteins Vps35, Vps26 and Vps29) mediates the recycling of membrane proteins to the Golgi complex or back to the cell surface. The association of the core Retromer complex with different sorting nexin (SNX) proteins provides Retromer diversity in terms of cargo recognition and trafficking pathways [158]. Retromer cargos include for instance postsynaptic neurotransmitter receptors [159,160] and the dopamine transporter DAT, a presynaptic protein required for dopamine clearance following release [161]. Although little is known about how aging influences Retromer efficiency, an age-dependent

decrease of most Retromer components is observed in the mouse cortex [162]. When Retromer-dependent processes are disrupted, many protein cargoes are misdirected to the degradative pathway, leading to improper recycling of for instance lysosomal cathepsins and neurodegeneration [163]. Recent studies in *Drosophila* suggest that presynaptic Retromer regulates SV recycling [164,165], although Vps35 depletion in primary hippocampal mouse neurons did not alter synaptic vesicle release or retrieval [166].

In the aging brain, the UPS is proposed to be modified in different ways, including alterations in the ubiquitin-conjugating and DUB enzyme landscape [167–170] and the expression or transport of proteasome subunits [171,172]. The accumulation of protein inclusions due to proteostasis failure can also clog and inactivate the proteasome [173]. This proteostasis feedback during aging makes it difficult to dissect primary causes, such as changes in UPS expression, from secondary consequences due to protein overload. In synaptosomal fractions from aged monkey brains, levels of ubiquitin-conjugating enzyme UBE2N increase with aging while UPS activity declines, potentially leading to an accumulation of ubiquitinated and misfolded proteins in neuronal terminals [170]. Similarly, proteasome activity is decreased during aging in *Drosophila*, and enhancement of neuronal proteasome function prolongs lifespan and delays age-related decline in memory and learning [174], although the background mechanisms are so far unknown. Little is known about age-related UPS changes and their impact on cognition in humans. A recent proteomic study on enriched synaptic fractions from human brain samples connected the levels of proteasome subunits to cognitive performance [135]. Here, proteasome subunit levels were decreased in subjects that, despite AD pathology, are cognitively unimpaired but elevated in aged individuals with low pathology but with impaired cognition. In conclusion, whereas most studies indicate age-related decline in proteasome function, the relationship between cognitive aging and impairment of the UPR are far from clear.

Conclusions and open questions

In recent years, we have gained fundamental insight into the degradative mechanisms involved in synaptic protein turnover and their relevance for neurotransmission. Both basal and stress-evoked pathways can damage synapse integrity and further research will be vital to uncover how synaptic components such as aged SVs are recognized and tagged for degradation. In addition, how do different regulators and signals such as neuronal activity, mTOR and ER-stress impinge on local degradative pathways in the synapse? How is the synaptic availability and activity of ubiquitin ligases and DUBs regulated, and how do all these different degradative pathways cooperate to sustain synapse integrity?

Much of what we know about aging comes from rodents, mostly from transgenic mouse model of age-related neurodegenerative diseases such as AD and PD. However, emerging studies show different, and often opposite, effects on the brain during neuropathology as compared with normal aging, indicating that neurodegenerative diseases are not a mere acceleration of aging. Whereas protein recycling in the brain decreases in aged, but otherwise healthy, mice, increased protein turnover is associated with aging in, for instance, mouse models of Alzheimer's disease [134]. Here, differential effects on degradative pathways might account for the discrepancy. For instance during pathology an unusual high amount of misfolded proteins can clog the proteasome and other degradative pathways. It is also important to recognize that individual synapses show high degree of diversity arising from molecular and morphological differences [175]. We know that region-specific vulnerability underlies pathological brain aging, with for instance the hippocampus primarily affected in Alzheimer's disease, the substantia nigra in Parkinson's disease and the ventral spinal cord in amyotrophic lateral sclerosis [176]. During physiological aging, changes in synaptic architecture, composition, and function also vary depending on brain region and synapse type [110,124]. In addition, not all brain areas experience the same age-related changes in degradative pathways [177], indicating that physiological brain aging is as diverse as pathological brain aging. Therefore, we have to take into account that population-averaging measurements might not be able to detect all changes that happen with age. What is underlying this differential vulnerability of neuronal and synaptic populations is unclear but likely depends on intrinsic differences between synapses, such as their activity, their turnover rates or dependence on, and availability of, certain proteolytic systems. Recent studies also suggest cell extrinsic factors to play a role in age-dependent synapse decline, including systemic factors such as microbial metabolites (reviewed in [178]). How environmental factors shape protein homeostasis in humans is still an open debate but non-pharmacological methods such as caloric restriction and physical exercise increase neuronal plasticity and resistance of neurons to age-related decline in humans, possibly via common pathways such as mTOR and autophagy [179]. Finding the molecular pathways involved in synapse aging will be essential to design future interventions to halt or even reverse brain aging.

Data Availability

No data were generated for this review.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRedit Author Contribution

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Abbreviations

AD, Alzheimer's disease; CMA, chaperone-mediated autophagy; mTOR, mechanistic target of rapamycin; PD, Parkinson's disease; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; ROS, reactive oxygen species; UPR, unfolded protein response; UPS, ubiquitin-proteasome system.

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