Chapter 13
Autophagic Pathology and Calcium Deregulation in Neurodegeneration

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Abstract Over the last decades, substantial efforts have been made towards understanding the key players underlying neurodegeneration. However, despite extensive research efforts, the exact molecular mechanism(s) remain unclear, and much less is certain about possible common target(s) amongst distinct age-dependent neurodegenerative disorders. Whilst the precise mechanism(s) underlying neurodegeneration amongst the different diseases remain to be determined, a number of cellular processes have been suggested to be involved in all of them, including protein accumulation and aggregation, oxidative stress, mitochondrial deficits, Ca^{2+} dyshomeostasis and impairments in lysosomal degradation pathways including macroautophagy. The various possible pathogenic factors are not mutually exclusive, and the aim of much current research is to elucidate the correlation between them to establish successful strategies in limiting the disease process. Here, we summarize recent data that pinpoint Ca^{2+} dyshomeostasis as a key player underlying neurodegeneration in the context of macroautophagy deregulation. We will provide a brief overview of recent work towards addressing how macroautophagy and Ca^{2+} deregulation may cause cellular dysfunction linked to the pathogenesis of several neurodegenerative disorders, with emphasis on Parkinson's disease (PD).

Keywords Calcium · Autophagy · Endoplasmic reticulum · Mitochondria · Acidic calcium stores · NAADP · Parkinson's disease · LRRK2

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Introduction

Neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD), which are characterized by progressive nervous system dysfunction, affect millions of people worldwide. There is still no cure for any of them, and treatment is directed towards alleviating some of the symptoms. Slowing the rate of neuronal loss may improve the quality of life for patients; however, this requires considerable knowledge about disease mechanism(s) and approaches able to modify disease course. In this respect, although the majority of neurodegenerative diseases are sporadic, the discovery of mutations that cause the pathological processes has significantly advanced our understanding of the molecular mechanisms involved, as it allows for the generation of cellular and animal models carrying the mutant gene. Hence, even though the brain areas that degenerate and the proteins implicated in each disorder are generally different, the above-mentioned diseases may converge on common cellular processes. The presence of intra-cytoplasmic aggregates within neurons as a hallmark of most neurodegenerative diseases suggests that protein misfolding and aggregation are central features of the pathophysiology [1]. Indeed, most of these pathologies are referred to as protein misfolding diseases [2]. In support of the importance of protein misfolding and aggregation in neurodegeneration, accumulation of tau in AD and tauopathies, α-synuclein in PD, mutant huntingtin in HD and TDP-43 and FUS in ALS seem to mediate neuronal toxicity [1].

Autophagy is a metabolically homeostatic process by which old and/or damaged organelles and protein aggregates are removed from the cell. Thus, the above-mentioned processes which may underlie neurodegeneration, including increased oxidative stress, mitochondrial dysfunction and protein aggregation may all result from impaired autophagic degradation. Indeed, apart from neurodegeneration, autophagic failure has been related to a wide range of additional pathological conditions including cancer and pathogen infection [3]. Alterations in autophagy seem a key event underlying neurodegeneration, and accumulation of autophagic structures is evident in diseased brains as well as in various animal and cellular models [4]. While environmental factors may further impair autophagic clearance, disease-linked mutations reveal possible pathways susceptible for therapeutic interventions. Indeed, studies from animal and cellular models of neurodegenerative diseases indicate a connection between proteins genetically linked to disease and autophagy impairment [5–16]. Therefore, many attempts aimed at treating these diseases are focused on the development of novel therapies towards promoting autophagic clearance. In this respect, pharmacological activation of autophagy has been shown to alleviate the toxicity associated with accumulation of aggregate-prone proteins [17].

Recently, Ca^{2+} handling mechanisms have been recognized to be crucial for proper cellular degradation, suggesting that the role of intracellular Ca^{2+} in regulating autophagy, and alterations in Ca^{2+} signaling in general, have to be considered towards gaining an understanding of the process(es) underlying neurodegeneration. Ca^{2+} homeostasis is essential for neuronal activity and viability. Ca^{2+} is in a

dynamic cellular equilibrium in the cell, and generally kept at low cytosolic concentrations, being either pumped out of the cell, buffered by cytosolic Ca^{2+} buffering proteins, or quickly taken up into a variety of intracellular organelles which act as intracellular Ca^{2+} stores. Highly controlled regulatory mechanisms ensure such normal Ca^{2+} handling, and perturbations lead to a vast array of pathologies. Hence, Ca^{2+} overload, manifested as an increase of cytosolic Ca^{2+}, has pleiotropic effects on a variety of intracellular events and eventually activates both necrotic and apoptotic cell death pathways [18]. There is now compelling evidence for dysfunctional Ca^{2+} regulation in several neurodegenerative disorders including PD [19], AD, HD [21] and ALS [22]. Therefore, Ca^{2+} channels may comprise promising neurodegenerative drug targets, and therapies based on channel blockers are being developed [23–25].

A possible crosstalk between dysfunctional Ca^{2+} handling, autophagy impairment and neurodegeneration may highlight additional therapeutic targets. However, more work is needed to dissect the links between Ca^{2+} and autophagic clearance mechanisms. In this chapter, we will first briefly review the current knowledge of the molecular events of autophagy. Next, we will give an overview of the regulatory role of Ca^{2+} in autophagy, summarizing the mechanisms involved in the dynamic equilibrium of Ca^{2+} uptake and release pathways with a focus on the different Ca^{2+} stores and fluxes as determinants of proper autophagic functioning. Finally, we will focus on the link between autophagic alterations and Ca^{2+} deregulation using PD as an example, and discuss the Ca^{2+}-dependent mechanisms believed to be implicated in the selective degeneration of dopaminergic (DA) neurons [19].

Autophagy

Autophagy likewise targets proteins, protein aggregates or entire organelles for lysosome-mediated degradation. It is important for balancing sources of energy at critical times in development and in response to nutrient stress, and it plays a key role in the homeostatic clearance of defunct or damaged organelles, proteins and protein aggregates [3, 26]. Autophagy normally proceeds at a low basal rate, but basal autophagy is especially high in neurons, and disrupting basal autophagic degradation causes neurodegeneration in mice [27, 28]. Depletion of different autophagic proteins in mice causes an accumulation of cytoplasmic protein inclusions accompanied by progressive deficits in motor function [27, 28].

There are at least three types of autophagy, depending on the delivery route of the material for degradation to the lysosomal lumen: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy [29, 30]. CMA is a selective mechanism responsible for the lysosomal degradation of soluble cytosolic proteins targeted with a consensus motif biochemically related to KFERQ [5]. In CMA, proteins complexed to chaperone proteins are targeted to the lysosomal membrane by binding to the CMA-receptor lysosome-associated membrane protein (LAMP) 2 A (LAMP2A), resulting in their translocation to the lysosomal lumen and subsequent

degradation. Defective CMA has been attributed to underlie different pathologies including lysosomal storage diseases and familial forms of PD [5]. Microautophagy is a mechanism by which a portion of cytoplasm is directly taken up by the lysosome itself through invagination of the lysosomal membrane, but its mechanisms and physiological functions are poorly understood [31]. Finally, a third form of autophagy is macroautophagy (hereafter referred to as autophagy), a process by which cytosolic constituents, including damaged organelles and aggregated proteins, are engulfed within newly formed specialized double-membrane vesicles called autophagosomes. Autophagosomes fuse either with late endosomes (LE) or directly with lysosomes, followed by the hydrolytic degradation of products in lysosomes and reformation of these organelles to maintain cellular degradative capacity. Disrupting any part of this process impairs autophagic flux, accompanied by the accumulation of autophagic substrates.

Autophagy Signaling Pathways

The signaling mechanisms leading to the activation of autophagy under nutrient starvation conditions have been extensively investigated [32]. However, these circumstances are rare under physiological conditions. Nevertheless, autophagy plays an essential role in the maintenance of normal homeostasis at both a cellular and organismal level, and can also be induced by several cellular stresses under normal nutritional conditions. Under normal non-starved conditions, autophagy is regulated by a broad range of extracellular factors including growth factors, cytokines and chemokines, suggesting that there is a mutual exclusive regulation of cell growth and autophagy [33]. These factors are especially important in neurons, because these cells depend almost exclusively on glucose to provide both energy and carbon chains for protein synthesis in an insulin-independent manner. Thus, autophagic responses in neurons may be more related to organelle damage or neuritic remodeling than to the generation of amino acids and energy.

The best known canonical autophagy regulator is the mammalian TOR kinase (mTOR), specifically complex1 (mTORC1, comprised of by mTOR, RAPTOR, GβL/mLst8, PRAS40 and DEPTOR). Given the existence of some mTOR-independent autophagy pathways, autophagy signaling has been divided accordingly into mTOR-dependent and mTOR-independent pathways. mTOR is a serine/threonine protein kinase that belongs to the phosphatidylinositol kinase-related kinase (PIKK) family. mTOR regulates the balance between anabolic (cell growth) and catabolic (autophagic degradation) processes and has recently come to light as a prime modulator of neurodegenerative diseases [34]. The mTORC1 complex is positively regulated by the small GTPase Rheb, which can be inactivated by the GTPase activating protein (GAP) formed by the heterodimer regulator tuberous sclerosis 1 and 2 (TSC1/TSC2). In response to amino acids, mTORC1 has been reported to be recruited to the lysosomal surface by Rag GTPases [35]. Such lysosomal localization seems to be required for activation of the complex by Rheb [36]. The active

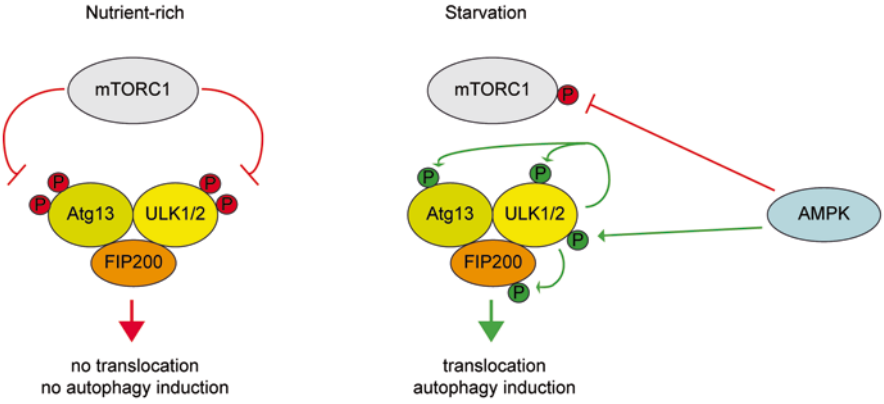

Fig. 13.1 Schematic overview of the regulation of autophagy induction by mTORC1 and AMPK signaling. *Left*: Under nutrient-rich conditions, activated TORC1 inhibits autophagy induction by inhibitory phosphorylation of ULK1/2 and Atg13, thereby inhibiting this complex. *Right*: Under starvation conditions, the mTORC1-dependent phosphorylation sites in ULK1/2 are rapidly dephosphorylated, and ULK1/2 can autophosphorylate itself and phosphorylate Atg13 and FIP200. Alternatively, activation of AMPK under distinct situations can induce autophagy by inhibiting mTORC1 through phosphorylation of a component within this complex. AMPK can phosphorylate and activate ULK1/2, with activation and translocation of the complex to the pre-autophagosomal membrane for autophagy induction. For further details see text

mTORC1 complex promotes cell growth and inhibits autophagy by inhibitory phosphorylation of the mammalian Atg1 orthologues ULK1 and ULK2 (ULK1/2) and Atg13 subunits [37] (Fig. 13.1). mTORC1 activity can be inhibited indirectly by another autophagy-related kinase, adenosine monophosphate-activated protein kinase (AMPK). As mentioned before, autophagy can also be controlled by several additional mTORC1-independent pathways. Direct induction of autophagy can be mediated, downstream of mTORC1, by the activation of AMPK in response to energy loss (an increase in AMP levels), involving various upstream regulators including LKB1 or an increase in cytosolic Ca^{2+} levels and concomitant activation of Ca^{2+}-calmodulin-dependent kinase kinase β (CaMKKβ) [38, 39]. Activated AMPK phosphorylates the ULK1/2 complex and directly induces autophagosome formation [40, 41]. Hence, both mTORC1-dependent and mTORC1-independent signaling pathways converge onto ULK1/2; mTORC1 and AMPK oppositely regulate ULK1/2 kinase activity by direct phosphorylation of the ULK1/2 complex on distinct sites [37, 42, 43] (Fig. 13.1). In both cases, ULK1/2 activation leads to autophosphorylation and stimulatory phosphorylation of both FIP200 and Atg13, which causes translocation of the entire complex to the pre-autophagosomal membrane and autophagy induction (Fig. 13.1).

Autophagic Steps

The autophagic pathway is comprised of distinct steps, including nucleation to generate a phagophore, elongation and formation of the autophagosome, autophagosome fusion with late endosomes or lysosomes, and finally degradation and recycling of macromolecules [44]. The source of the autophagosomal membranes is still controversial, although it is generally accepted that the ER membrane provides the major source of autophagosome membrane [45]. More recently, mitochondria [46, 47], plasma membrane [48], recycling endosomes [49] and the trans-Golgi network (TGN) [50, 51] have all been shown to be cellular sources for autophagophore formation, even though it remains unclear how much of the autophagosome membrane comes from such distinct sources, and under which conditions.

A crucial element for the initiation of the isolation membrane is the transient association of a large core complex including Vps34-Beclin1 [29, 52]. The protein AMBRA1 has been proposed as the link between ULK1/2 and the Vps34-Beclin1 complex [53]. ULK-dependent phosphorylation of AMBRA1 might lead to the release of the Vps34-Beclin1 complex from the dynein motor complex tethered to the cytoskeleton to allow translocation of the core complex to the autophagosome formation-related region [53]. Alternatively, ULK may directly phosphorylate Beclin1 to enhance Vps34 lipid kinase activity [54]. Vps34 is a class III phosphatidylinositol-3-kinase, and the lipid phosphatidylinositol-3-phosphate (PI3P) seems important for the initiation of the autophagosome membrane, with PI3P-enriched membranes recruiting and activating effector proteins containing FYVE or PX PI3P-binding domains [55]. The isolation membrane subsequently elongates, sequestering the cargo for degradation, and finally closes off to form a double-membraned vesicle called the autophagosome. The elongation process is regulated by two ubiquitination-like reactions, the Atg12/Atg5 conjugation and the conjugation of microtubule-associated protein 1 light chain 3 (LC3) to the lipid anchor phosphatidylethanolamine (the autophagosome-associated LC3-II form) [44]. The action of those two ubiquitin-like conjugation systems is modulated by Atg7. The Atg5/12 complex is further associated with Atg16L and LC3, but dissociates from the vesicle once it is fully formed [56]. In contrast, LC3, which is diffuse in the cytoplasm under normal conditions (LC3I), is bound to autophagic membranes (LC3II) upon the above-mentioned conjugation reaction and is classically used as a marker for autophagy (Fig. 13.2). Autophagosomes are then transported on microtubules from the cell periphery to the perinuclear region towards the microtubule-organizing center in a dynein/dynactin-dependent manner [57]. During maturation, the outer membrane of the autophagosome fuses with late endosomes, generating a hybrid organelle called the amphisome, or directly fuses with lysosomes to form an autolysosome (Fig. 13.2). Fusion events during autophagic maturation are similar to endosome-lysosome fusion events, where kiss-and-run, complete fusions or fusion mediated through tubules have been described [58]. Several protein complexes and signaling pathways are involved in the maturation of autophagosomes and their delivery to, and fusion with, lysosomes. Finally, lysosomal hydrolases degrade the

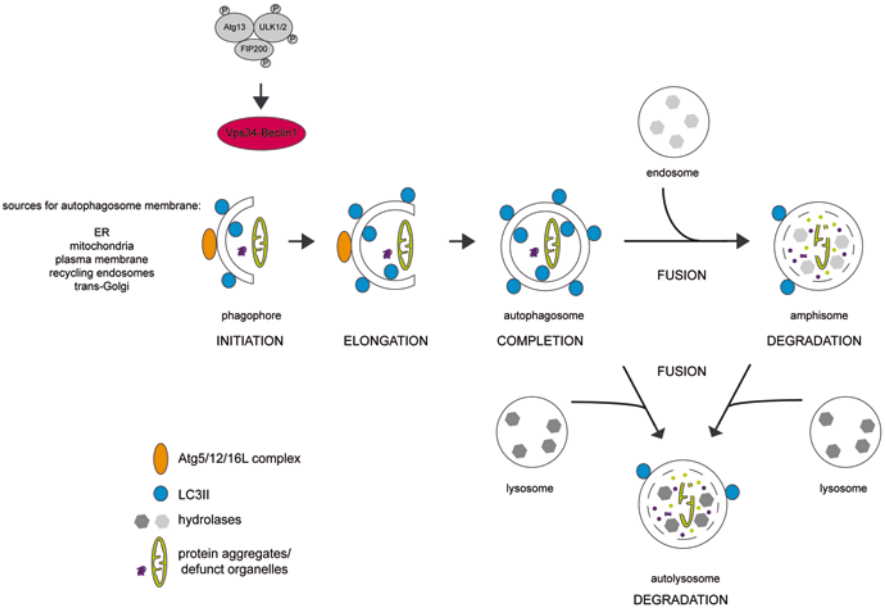

Fig. 13.2 Autophagic steps. Activation of the ULK1/2 complex leads to activation of Vps34-Beclin1 complex, followed by transient association with the isolation membrane and phagophore formation. The Atg5/12/16L complex and LC3II promote elongation and enwrap cytosolic cargoes including defunct organelles such as mitochondria and protein aggregates, finally resulting in the formation of the autophagosome. Autophagosomes then either fuse with endosomes to form amphisomes, or with lysosomes to form autolysosomes, and hydrolases in the interior begin to degrade internal membrane and luminal cargoes. For further information see text

content and the inner membrane of the autophagosome/amphisome. After this process, lysosomal function is restored again by formation of proto-lysosomes budding off from tubules emanating from the autolysosomes. These proto-lysosomes have been reported to be non-acidic and LAMP1 positive [59].

Ca^{2+} as an Autophagy Regulator

The spatiotemporal pattern of Ca^{2+} signaling is crucial for the specificity of cellular responses such as autophagy. Ca^{2+} has an important regulatory function in key steps of the autophagic pathway including during homotypic and heterotypic fusions of late endosomes and lysosomes and during lysosomal reformation [60], analogous to the well-described role of Ca^{2+} in regulated secretion. Moreover, autophagosome formation and maturation may be regulated by Ca^{2+} from different membrane sources [61, 62]. However, Ca^{2+} has been better recognized as an important player for autophagic signaling (see above). Intracellular Ca^{2+} has been proposed to be a regulator of autophagy, even though it is unclear whether there is positive [63, 64]

or negative [65–68] regulation. Dual effects have been reported and may depend on several circumstances such as the spatial and temporal parameters of Ca^{2+} signaling, the source of the Ca^{2+} and the overall cellular state [69].

Changes in intracellular Ca^{2+} caused by the action of Ca^{2+}-mobilizing agents have been reported to lead to autophagic alterations. The first evidence for a positive Ca^{2+}-mediated regulation of autophagy was reported by Jäättelä's group, as Ca^{2+}-mobilizing agents were found to activate the CaMKKβ/AMPK pathway, causing an induction of autophagosome formation [63]. These data suggested that Ca^{2+} may act as a trigger for autophagy, further supported by findings that the ionophore ionomycin, which raises cytosolic Ca^{2+} levels, induced autophagy, whilst a membrane-permeable Ca^{2+} chelator blocked the effects on autophagy. However, Ca^{2+} may also negatively regulate autophagy through acting as a bioenergetic sensor and inhibiting the catabolic mechanism of autophagy under normal cellular growth conditions. Therefore, a coherent understanding of the dual role of Ca^{2+} in regulating autophagy requires an understanding of the precise intraorganellar location of Ca^{2+}, the role which Ca^{2+} plays at these organelles, and the precise cellular status [69].

Neuronal Ca^{2+} entry from the extracellular space can be either mediated by voltage-dependent Ca^{2+} channels or ligand-activated Ca^{2+} influx [20]. Pumping Ca^{2+} out of the cytosol is an energetically costly process, as the removal is carried out by Ca^{2+}-ATPases localized on the plasma membrane (PMCAs) or on intracellular Ca^{2+} stores, such as the endoplasmic reticulum Ca^{2+} pumps named SERCA (sarco/endoplasmic-reticulum Ca^{2+}-ATPase) [70]. Several cellular compartments that have functional Ca^{2+}-release channels and pump mechanisms are essentially capable of acting as Ca^{2+} stores. The ER is the main and best-characterized Ca^{2+} store within the cell, whilst mitochondria can act as additional stores to buffer intracellular Ca^{2+}. At the same time, Ca^{2+} is also important in the normal ER and mitochondrial function, affecting protein folding and mitochondrial metabolism, therefore modulating autophagy. In addition to the ER and mitochondria, a range of acidic organelles, which include endosomes, lysosomes and lysosome-related organelles such as secretory granules also serve as significant Ca^{2+} stores in mammals [71]. Intraluminal Ca^{2+} in acidic stores is crucial for the functioning of the degradative pathway, as chelation of luminal Ca^{2+} blocks membrane fusion [72–74]. Therefore, the overall intraluminal Ca^{2+} concentrations and dynamics between the different Ca^{2+} stores are crucial for autophagy modulation.

ER and Mitochondrial Ca^{2+} and Regulation of Autophagy

The ER produces, delivers and folds newly synthesized proteins in a continuous intracellular network, but also it acts as the major intracellular Ca^{2+} store, setting up cytosolic Ca^{2+} signals. Whilst SERCA transports Ca^{2+} from the cytosol to the ER lumen in an energy-dependent manner [75], the main Ca^{2+}-release channels in the ER are the inositol 1,4,5-trisphosphate (IP_3)-receptor (IP_3R) [76] and the ryanodine-receptor (RyR). Ca^{2+}-binding proteins in the lumen of the ER, such as

Ca^{2+}-dependent chaperones like calnexin and 78-kDa glucose-regulated protein/immunoglobulin heavy chain binding protein (GRP78/BiP) further contribute to Ca^{2+} handling in the ER [77]. IP_3R are expressed in all cell types, while RyRs are mainly expressed in neurons and muscle. IP_3 and cyclic adenosine diphosphate-ribose (cADPR) mobilize Ca^{2+} from the ER through activation of these receptors [78, 79], generating local Ca^{2+} signals. The subcellular localization of these receptors, together with their regulation by cytosolic Ca^{2+} and regulatory proteins further contribute to the establishment of highly specific and localized Ca^{2+} signals.

A role for Ca^{2+} as a negative regulator of basal autophagy has been proposed by several reports and implicates the IP_3R. Rubinsztein's group reported that inhibitors of inositol monophosphatases, leading to depletion of IP_3 levels, act as novel chemical inducers of autophagy [65]. These data would suggest that IP_3R-mediated release of Ca^{2+} from the ER, presumably coupled to an increase in cytosolic Ca^{2+} levels, negatively regulates autophagy. In accordance with this concept, they further found that L-type Ca^{2+} channel antagonists induce autophagy through a complex mTORC1-independent signaling pathway impacting upon calpain [66] (Fig. 13.3). Calpains are cytosolic Ca^{2+}-dependent cysteine proteases that have been shown to participate in apoptotic cell death mechanisms [80], but also in the degradation of Ca^{2+} extrusion channels, thereby contributing to increased Ca^{2+} overload [81] and providing a positive feedback loop for autophagy inhibition.

The apparently contradictory data regarding cytosolic Ca^{2+} in stimulating or inhibiting autophagy may be reconciled if considering the combination of alterations in cytosolic Ca^{2+} levels, alterations in intraluminal Ca^{2+} stores and the bioenergetic status of cells. For example, intraluminal Ca^{2+} is crucial for ER function, as ER Ca^{2+} depletion induces ER stress and ER Ca^{2+} overload compromises proteostasis as well. Conversely, normal ER function is necessary for proper ER Ca^{2+} handling, as ER stress disrupts luminal Ca^{2+} homeostasis, causes the accumulation of misfolded proteins and initiates the unfolded protein response (UPR) [82]. The UPR response is activated to restore normal cellular conditions through activation of signaling pathways that reduce protein synthesis and increase protein folding capacity. Autophagy is activated in parallel to the UPS as another pro-survival mechanism, as autophagy has been shown to inhibit tunicamycin- or thapsigargin-mediated cell death [83]. However, the crosstalk between ER stress-induced autophagy and ER stress-induced Ca^{2+} release remains largely unanswered. It has been proposed that ER-mediated Ca^{2+} release induced by ER stress may cause CaMKKβ-dependent activation of AMPK to promote Ca^{2+}-mediated autophagy [63]. Other Ca^{2+}-dependent kinases may be involved in the positive regulation of autophagy as well, including the Ca^{2+}-regulated death-associated protein kinase (DAPK), whose role in ER-stress has been widely reported [84], or PKCθ [85].

The interplay between mitochondria and ER in zones of close contact termed MAMs (mitochondria-associated ER membranes) is crucial for cellular Ca^{2+} handling as well as for mitochondrial dynamics (86). Mitochondria are important Ca^{2+} stores and contain several Ca^{2+} channels, such as the voltage-dependent anion channel (VDAC) and Ca^{2+} uniporters which drive Ca^{2+} entry at high cytosolic Ca^{2+} concentrations reached in these areas. Ca^{2+} signaling regulates mitochondrial traf-

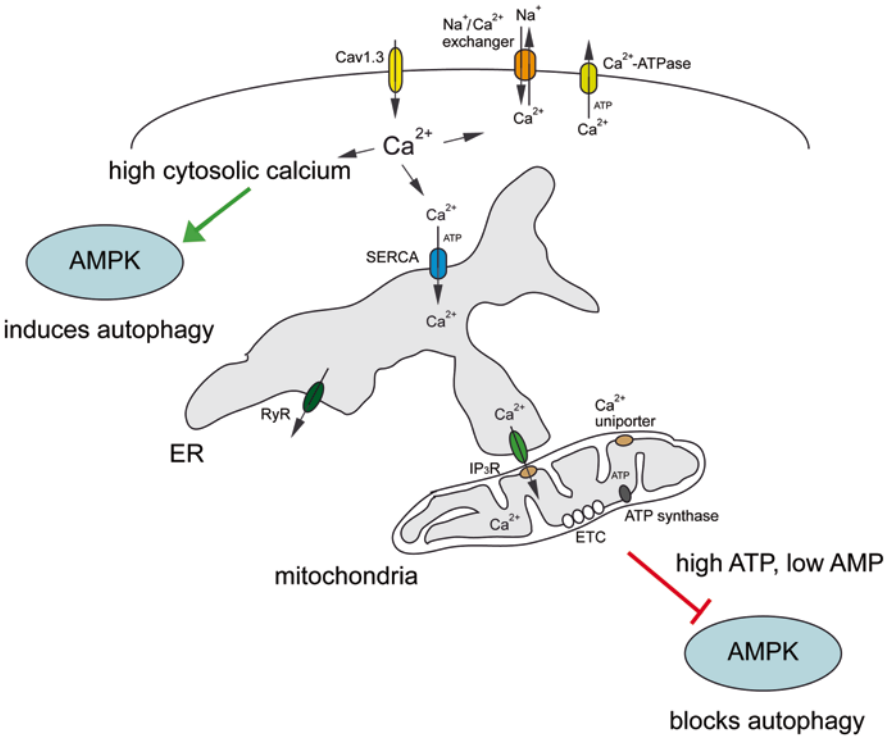

Fig. 13.3 Possible roles for Ca^{2+} in regulating autophagy. Ca^{2+} entry into cells is quickly controlled, either by extrusion or by uptake into the ER. IP_3R mediate a constitutive transfer of Ca^{2+} from the ER to mitochondria, which promotes ATP production, inhibiting AMPK and keeping autophagy to a minimum. Blocking such Ca^{2+} transfer, with the resultant increase in AMP, will stimulate autophagy. On the other hand, increasing cytosolic Ca^{2+} levels, e.g. by Ca^{2+}-mobilizing agents which may target a variety of intracellular Ca^{2+} stores, increases the cytosolic Ca^{2+} concentration. This causes autophagy induction through various pathways, including CaMKKβ and AMPK. For further details see text

ficking and dynamics through the regulation of mitochondrial fusion and fission reactions [87]. Furthermore, release of Ca^{2+} from ER IP_3R is important for mitochondrial activity and bioenergetics, as several mitochondrial dehydrogenases of the Krebs cycle are regulated by Ca^{2+}. Hence, under normal nutritional conditions, constitutive IP_3R Ca^{2+} transfer from ER to mitochondria inhibits autophagy for maintenance of cellular metabolic homeostasis (Fig. 13.3). In the absence of such Ca^{2+} transfer, AMPK is activated due to a decrease in mitochondrial ATP production and concomitant increase in AMP levels, causing AMPK-dependent but mTORC1-independent autophagy induction (67). Conversely, abnormal mitochondrial Ca^{2+} overload leads to the depolarization of the mitochondrial membrane potential with a concomitant decrease in ATP synthesis, autophagy induction and eventually the opening of the permeability transition pore and cell death. Therefore, a tight control and crosstalk of Ca^{2+} dynamics in distinct intracellular organelles, combined

with cellular energy status, may explain the opposing effects of Ca^{2+} on autophagy. Under normal conditions, Ca^{2+} may play a role as a negative regulator of autophagy, but under conditions of ER or mitchondrial stress conditions, Ca^{2+} may activate autophagy to help the cell to cope with a variety of potentially damaging events related to those intracellular organelles. Future experiments aimed at detecting Ca^{2+} changes in the cytosol, the ER and mitochondria will be necessary to dissect the precise events underlying the dual regulation of autophagy by Ca^{2+} signaling.

Acidic Ca^{2+} Stores and Regulation of Autophagy

Endosomes pinching off from the plasma membrane are thought to contain high levels of Ca^{2+} due to the elevated concentrations of this ion in the extracellular fluid, whilst only having a slightly acidic pH (pH 5.9–6.3) [88]. It has been proposed that the uptake of H^+ into endosomes mediated by the proton-pumping ATPase must be balanced to maintain electroneutrality, and that this may happen by Ca^{2+} efflux via endosomal Ca^{2+} channels [89]. Even though direct measurements of luminal lysosomal Ca^{2+} concentrations are technically difficult, the average free Ca^{2+} concentration in lysosomes has been reported to be in a similar range as that of the ER [90], with an average free Ca^{2+} concentration in the range of 500 μM [73]. Whilst less acidic (pH ~ 6.6), the Golgi apparatus also deserves attention as significant intracellular Ca^{2+} store. It contains substantial levels of Ca^{2+}, Ca^{2+} release channels such as the IP_3R as well as Ca^{2+}-binding proteins [91].

Several Ca^{2+}-permeable channels are present on acidic organelles. IP_3R and RyR have been found to be located on acidic Ca^{2+} stores [92]. However, nicotinic acid adenine dinucleotide phosphate (NAADP) has been postulated to be the most powerful endogenous Ca^{2+}-mobilizing messenger known to date and the major regulator of Ca^{2+} release from endolysosomes [93–95]. Two different families of channels have been proposed to be intracellular NAADP targets, including members of the transient receptor potential (TRP) superfamily, particularly the mucolipins (TRPMLs), which are found on acidic stores such as lysosomes and endosomes [96]. Mutations in the TRPMLs channels are responsible for several lysosomal-storage disorders [96]. There are three TRPMLs, displaying differential albeit partially overlapping localization within the endo-lysosomal compartment [71]. TRPML1 has been proposed as the Ca^{2+} channel responsible for the local release of Ca^{2+} crucial for vesicular fusion reactions within the endo-lysosomal system [97]. There is evidence that TPRML1 may be an NAADP target, whilst other studies indicate that $PI(3,5)P_2$, rather than NAADP, may regulate the channel [98, 99]. Interestingly, TRPML1 has been reported to co-immunoprecipitate with the other family of Ca^{2+} channels, the two pore channels (TPCs) [100].

Two TPC isoforms are present in humans. TPC1 localizes to both endosomes and lysosomes, whereas TPC2 is predominantly lysosomal [101]. There have been several independent studies indicating that the TPCs comprise primary NAADP targets [71]. NAADP may not directly bind to TPCs, but rather indirectly through binding

to currently unidentified low-molecular weight proteins which interact with TPCs. Inhibition of the NAADP-mediated Ca^{2+} response occurs upon downregulation of the TPCs or when overexpressing TPCs with a mutation in the pore-forming region [102]. Even though controversy has recently been raised as to the ionic specificity of the TPCs, which have been suggested to behave as a sodium-selective channels activated by $PI(3,5)P_2$, rather than by NAADP [103, 104], the vast majority of studies indicate that the TPCs can conduct Ca^{2+}.

In analogy to ER-mitochondrial contact sites, membrane contact sites between ER and the endo-lysosomal system have been suggested to correspond to NAADP trigger zones [101]. This interaction has been proposed to be similar or maybe shared with that which mediates cholesterol exchange between the ER and late endosomes [105], and is mediated by the interaction of Rab7 and RILP on the late endosome and the VAMP-associated ER protein (VAP) through ORP1L. In this manner, release of Ca^{2+} from the endo-lysosomal compartment, triggered by NAADP, may evoke local cytosolic Ca^{2+} signals which can by amplified into global Ca^{2+} waves through Ca^{2+}-induced Ca^{2+} release (CICR) from the ER [106].

Several cellular processes are regulated by Ca^{2+} release from acidic stores. NAADP-mediated Ca^{2+} release has been related to cell differentiation and induction of neurite outgrowth [107], and NAADP has also been shown to increase the levels of autophagic markers in cultured astrocytes [104], HEK293 and PC12 cells [13]. Indeed, the rise in free cytosolic Ca^{2+}, likely caused by amplification through CICR from the ER has been shown to induce autophagy by activation of CaMKKβ-dependent activation of AMPK in a mTORC1-independent manner [13], and therefore seems to positively regulate autophagy induction (Fig. 13.3). However, NAADP has also been reported to transiently alkalinize lysosomal pH [13, 94], thereby affecting the recruitment of Rab7 to autophagosomes and disrupting autophagic flux [108]. These data indicate complex effects of NAADP on autophagy regulation, subject to further studies.

Autophagic Pathology and Calcium Deregulation in PD

Whilst the role of Ca^{2+} homeostasis for proper autophagic degradation is increasingly recognized, the precise alterations in Ca^{2+} handling and their effects on autophagic degradation in the context of various neurodegenerative disorders remain to be deciphered. Possibly the links are best understood in PD, where both autophagic failure and Ca^{2+} dysregulation have been extensively shown to play crucial role(s) in the pathomechanism(s) underlying the disease.

PD is characterized by the progressive degeneration of dopaminergic (DA) neurons in the pars compacta of the substantia nigra (SNpc), together with the presence of intraneuronal inclusions called Lewy bodies and abnormal dystrophic neuronal processes called Lewy neurites in surviving neurons [109]. While the exact pathological mechanisms underlying PD remain largely unclear, with most cases being

sporadic or idiopathic, having no apparent familial heritage nor relation to a defined environmental cause, several familial mutations in a variety of genes, with either autosomal-recessive (parkin, PINK1, DJ-1) or autosomal-dominant (α-synuclein, LRRK2) inheritance have been reported for approximately 10% of PD cases.

Autophagic Failure in PD

Compelling evidence suggests a deficit in autophagic clearance in PD. Increased number of autophagic structures have been found in PD patients, animal and cellular models of PD [110]. Another piece of evidence linking autophagy to PD comes from the reported effects of α-synuclein overexpression in blocking autophagy [12]. In addition, α-synuclein and UCHL-1 mutants have been reported to block CMA [5]. Conversely, pharmacological activation of autophagy has been shown to alleviate the toxicity associated with mutant α-synuclein *in vitro* and *in vivo* [34]. Other PD-related proteins have been related to altered autophagic flux as well. For example, the absence of DJ-1, PINK1 or Parkin has been shown to impair basal autophagic flux and cause accumulation of autophagic markers, mitochondrial fragmentation and depolarization in human DA cells [111, 112]. LRRK2, related to both familiar and sporadic PD has been closely related with autophagy as well.

Neuronal Ca^{2+} Signaling and PD

Currently one of the best working models to explain the selective degeneration of DA neurons in PD relates to perturbations in Ca^{2+} homeostasis [19, 113]. Adult SNpc DA neurons are autonomously active, generating action potentials in the absence of synaptic input, as do many others neurons. However, whilst most neurons use exclusively monovalent cation channels to drive this pacemaking activity, SNpc DA neurons also employ L-type Ca^{2+} channels that allow Ca^{2+} to enter the cytoplasm [114]. Since the spatiotemporal pattern of Ca^{2+} signaling is crucial for the specificity of cellular responses, Ca^{2+} must be under a tight homeostatic control that requires energy. Therefore, Ca^{2+} must be extruded by ATP-dependent processes, either pumped out or rapidly sequestered by intracellular organelles. As previously explained, both processes are mediated by ATP-dependent pumps and exchangers [115, 116]. This additional energy requirement and resultant energy production via mitochondria can generate oxidative stress and production of reactive oxygen species. In addition, the increase in Ca^{2+} uptake by mitochondria from the ER through IP_3R may lead to an increase in the basal generation of oxidant stress in the mitochondria of SNc DA neurons, increasing SNc DA neuron vulnerability [114, 117]. Interestingly, Ca^{2+} channel blockers which have demonstrated good blood-brain barrier permeability and a long record of safe use in humans have been reported to have positive results in rodent PD models [114, 118].

LRRK2: A Possible Link between Altered Ca^{2+} Homeostasis and Autophagy

Autosomal-dominant mutations in LRRK2 are the most common genetic cause of late-onset PD [119]. Approximately 5–8 % of European individuals with a first-degree relative with PD carry mutations in LRRK2. Importantly, variations in LRRK2 increase PD risk, indicating that LRRK2 is an important player for both genetic and sporadic forms of the disease [120].

There are indications that LRRK2 plays an important role in autophagy [110]. PD-linked mutations in LRRK2 seem to cause an impairment in autophagic degradation with a concomitant accumulation of undegraded material, lysosomal-like structures and lipid droplets in cell as well as animal model systems [8, 13, 15, 121–124], even though positive regulatory effects on autophagy have been described as well [125] which warrant further investigations. Deficiencies in Ca^{2+} handling in the presence of autosomal-dominant mutations in LRRK2 have also been reported, leading to mitochondrial depolarization and altered mitophagy [126, 127].

Our recent studies indicate that mutant LRRK2 causes release of Ca^{2+} from acidic stores which activates the CaMKKβ/AMPK pathway and causes autophagy induction, whilst simultaneously partially alkalinizing endolysosomal stores, provoking a possible defect in endolysosomal homeostasis and a defect in autophagic clearance [13]. These events seemed to be mediated by NAADP, as they can be mimicked by lysosomal Ca^{2+} mobilization through NAADP and reverted by an NAADP antagonist or by overexpression of dominant-negative TPC2 receptor constructs. NAADP-mediated release of Ca^{2+} from the endolysosomal compartment can cause a partial alkalinization of acidic stores and induce lipid accumulation, which may occur secondarily to Ca^{2+} release [94]. The deregulation of intraluminal Ca^{2+} levels may then be responsible for the observed changes in lysosomal morphology, distribution and degradative capacity, as described for mutant LRRK2-expressing cells [13, 15, 121, 124]. Indeed, deregulation of endolysosomal Ca^{2+} homeostasis has been proposed as a primary cause of the lysosomal-storage disorder Niemann Pick C1, where the reduced lysosomal Ca^{2+} results in a block in endocytic trafficking to lysosomes [73]. Our recent work further indicates that pathogenic LRRK2 regulates the activity of Rab7, a master regulator of late endocytic membrane trafficking involved in endolysosomal and autophagosome-endolysosomal fusion events [128]. Given the recently reported links between Rab7 and TPC2 [108, 129], further epistasis-type studies are warranted to determine whether LRRK2 regulates Rab7 to modulate NAADP-mediated Ca^{2+} release from endolysosomal stores, or whether LRRK2 regulates NAADP-mediated Ca^{2+} release which then may modulate Rab7 activity for downstream endolysosomal fusion events (Fig. 13.4).

In either case, there is convincing evidence to support the hypothesis that deficiencies in Ca^{2+} homeostasis and protein degradation are crucial elements in the neurodegenerative process underlying several age-dependent neurodegenerative diseases. Whilst Ca^{2+} has been consistently showed to regulate autophagy, the positive or negative link between these two mechanisms in the context of

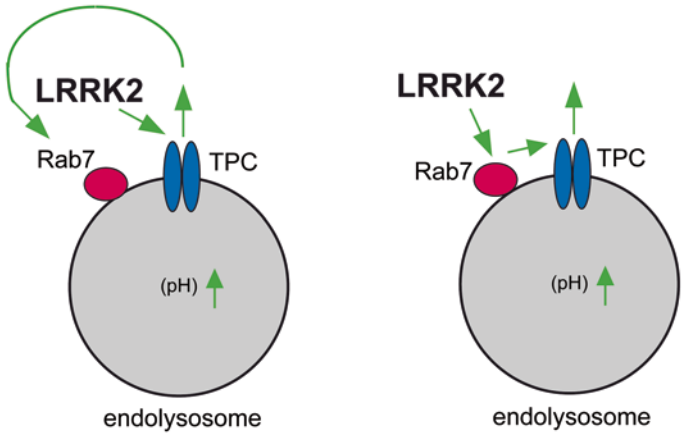

Fig. 13.4 Possible models for the link between pathogenic LRRK2, TPC channels and Rab7. *Left*: LRRK2 may regulate TPC channels, and the resultant alterations in endolysosomal Ca^{2+} exit may regulate Rab7. *Right*: LRRK2 may regulate Rab7 activity, which in turn may modulate TPC channels to alter Ca^{2+} release from endolysosomal stores. For further details see text

neurodegeneration and cellular status remains to be further elucidated, which may help to define novel therapeutic targets.

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