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The impact of autophagy on peripheral synapses in health and disease

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1. ABSTRACT

Alterations of autophagy have been linked to several peripheral nervous system diseases, such as amyotrophic lateral sclerosis and Charcot-Marie-Tooth disease. Modulation of autophagy by metabolic or pharmacological interventions has been increasingly recognized as a strategy to fight many of these disorders. Cellular processes that are aberrant in case of impaired autophagy and that might lead to these diseases belong to three different categories: (1) clearing of protein aggregates, (2) regulation of vesicle and cargo turnover, and (3) disposal of damaged mitochondria. This review summarizes the present literature that addresses both, the impact and mechanisms of autophagy on the health of the peripheral nervous system and treatment proposals for human disorders associated with impaired autophagy.

2. INTRODUCTION

A chemical synapse (in the following called synapse) is the point of contact between a presynaptic neuron and a postsynaptic cell, which itself can be a neuron, or other targets like muscle or gland cells. Structure and function of such synapses have been recently reviewed (1-3). Pre- and postsynapse are separated by a synaptic cleft that is usually a few dozen nanometers in width and exhibits tightly regulated spacing (4). This is a prerequisite for the principal function of synapses, which is the fast, graded, and reliable signal transmission from the pre- to the postsynaptic compartment. In vertebrates, synapses are present in the central and the peripheral nervous systems and they mediate a plethora of physiological functions. These range from learning and memory over voluntary muscle contraction to the control of heartbeat, glands, and digestion. Although plastic in nature to adapt to an ever changing environment, synapses are often maintained for extended time periods, as can be seen in the cases of long-term plasticity or of the nerve-muscle synapse, the neuromuscular junction (NMJ) (1), which may be nourished for the entire lifespan of an organism. This combination of physiological importance, high-speed signal transmission, and plastic and yet reliable maintenance renders synapses uniquely well controlled and at the same time delicate sites. Furthermore, synapses are mostly located on axons and dendrites, and therefore far away from the cell body with its transcription-translation machinery. Thus, due to the slow pace of axoplasmic transport (5,6), arrival at the synapse of newly made cytoskeletal filaments, secretory proteins, membrane receptors, and organelles typically takes hours to days. This is clearly beyond the physiological needs of an active synapse making tight control of local protein turnover and subcellular localization essential for proper synaptic function. While synaptic protein localization involves activity-dependent receptor recycling (7-11), protein turnover is determined by long-distance delivery of proteins from the perikaryon (12,13) as well as local RNA translation (14) and protein degradation (15). The latter uses three major proteolytic pathways, which are the ubiquitin proteasome system, lysosomal degradation, and autophagy. As reviewed recently (16-18), accumulating evidence shows that autophagy is critical for the maintenance of synapses

in the central nervous system and that both impairment as well as excessive autophagy can be detrimental for synapses. This article focuses on the role of autophagy in the peripheral nervous system with particular emphasis on neuromuscular transmission and related diseases.

3. AUTOPHAGY

Autophagy is a major proteolytic pathway responsible for degrading various targets such as proteins, organelles or lipids. Depending on the size, origin and other characteristics of the target, one can discriminate between several modes of autophagy which use different pathways to the lysosome for degradation (19,20): chaperone-mediated autophagy (21,22), microautophagy (23), and macroautophagy (24,25). Our review will primarily discuss macroautophagy in peripheral synapses and in the following text it will be referred to as "autophagy". Autophagy involves the nucleation of a double-membrane autophagic carrier called phagophore or isolation membrane which wraps around its target, elongates and closes to form the autophagosome (26). The origin of this membrane is still under debate but appears to include the ER or the Golgi complex. The autophagosome fuses with target structures (endosomes, defunct organelles, cytoplasmic aggregates) to form an amphisome (26,27). This amphisome merges with the lysosomal compartment to ensure protein degradation and the release of amino acids. Autophagosome formation and maturation is dependent on autophagy genes (Atg) and their assembly into large protein complexes. For a detailed review of molecular machinery the reader is referred to Russel *et al*. 2014 (28).

Although there is always a basal level of autophagy, its induction is tightly coupled to energy demand, lack of nutrients and removal of damaged organelles post pathophysiological stimuli. The regulation of autophagic pathways upon energy demand is brought about by mTORC1, known as principal regulator of cellular energy homeostasis, acts as a major switch in autophagy. mTOR which is the mammalian orthologue of the protein kinase TOR in yeast, controls many general cellular processes like mRNA translation or cell growth but fulfills special tasks as well. In the post-synaptic cellular partner of NMJ i.e. skeletal muscles, mTOR regulates hypertrophy as well as myotube fusion (29,30). It is assembled into two different complexes in mammals, the mTORC1 complex consisting of the catalytic subunit of mTOR, PRAS40 (proline-rich Akt substrate of 40kDa), GβL (G protein β-subunit-like protein) and raptor (regulatory associated protein of mTOR), and mTORC2 where raptor is replaced by rictor (mAVO3) (31,32). The cellular stress and energy/nutrient status regulates mTORC1 by either inhibiting the complex or activating it. Lack of growth factors or nutrients for example inhibits mTORC1, leading to autophagy induction while extracellular signals such as growth factors activate mTORC1, thereby suppressing autophagy (19,20).

Upon activation of mTORC1 under nutrient-rich conditions, its direct interaction with the ULK1 complex inhibits Atg13 and ULK1 kinase activity. Conversely, dissociation of mTORC1 from the complex, which can be mediated by e.g. rapamycin or nutrient deprivation, leads to the formation of autophagosomes (20). Besides controlling autophagy via the formation of autophagosomes, the mTor pathway also affects the expression of autophagy related genes such as LC3 in skeletal muscle. Indeed, the mTor-dependent change of Akt activity status leads to the inactivation/activation of Foxo3, a transcription factor responsible for the expression of a set of autophagy-driving genes, including LC3, Gabarapl1, Atg12, atrogin-1 and MuRF1 (33-35). Atrogin-1 and MuRF1 are E3 ubiquitin ligases belonging to the group of atrogenes, a class of proteins involved in atrophy of skeletal muscle tissue (36,37). Atrogin-1 as well as MuRF-1, are known to be involved in the ubiquitination of proteins targeted to the ubiquitin-proteasome system but recent findings suggest MuRF1 to be involved in selective autophagy as well (38,39). In selective autophagy, target proteins or organelles are ubiquitinated by E3 ligases and targeted to newly formed phagophores. This process relies on specific receptors, so called autophagy receptors and special adapter proteins such as ALFY (autophagy-linked FYVE protein) (40), Nbr1 (41) and p62 (Sequestome 1/SQSTM1). The autophagy receptors contain a LC3-interacting region (LIR) and an ubiquitin-associated (UBA) domain, important for the shuttling of ubiquitinated cargo to the autophagosome. The autophagy receptor binds the target protein via its UBA domain and connects it to the autophagosome by interacting with LC3 (42). Once the cargo is bound to the phagophore, the autophagosome matures by fusing with the lysosome for degradation. This process was not only found for intracellular proteins but also for transmembrane receptors, mitochondria (mitophagy), endoplasmic reticulum (ERphagy) or bacteria and viruses (xenophagy) (39,40,42,43). As mentioned earlier, autophagy is active under basal conditions as well and facilitates cytoplasmic waste clearance to avoid pathophysiological conditions. Consequently, failure of such clearance leads to cellular impairment. As will be discussed below, autophagic clearance of protein aggregates appears to be a key factor in the context of various peripheral synaptic diseases.

3.1. Autophagy-related diseases linked to protein aggregates

Among various peripheral synapses, autophagy affects neuromuscular function by impairing the integrity of motor neurons, Schwann cells, and muscle fibers. Similar to the importance of autophagy in central nervous system synapses, ample evidence from clinical research as well as animal models for peripheral neuropathies suggests that autophagic dysfunction leads to an imbalance between proteostasis and accumulation of

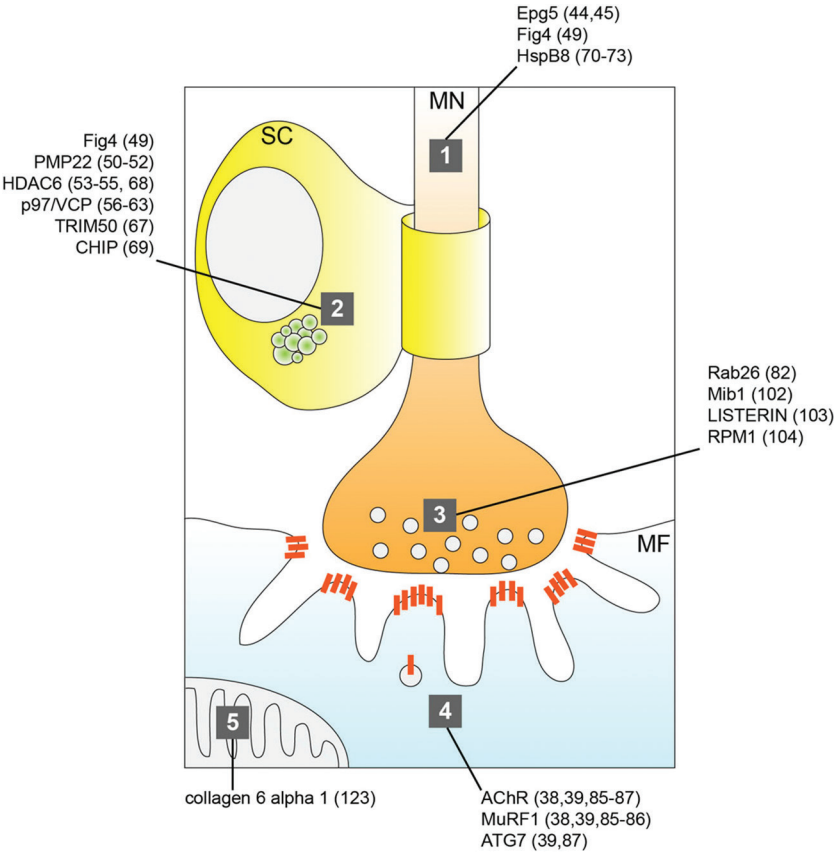

Figure 1. Involvement of autophagy in the peripheral neuromuscular complex. To date, it is known that alterations of autophagic processes are linked to a series of peripheral nervous system diseases. Upon impaired autophagy the major identified pathologies are (1) vacuolization, hypomyelination, and degeneration of peripheral neural axons, (2) inefficient clearing of protein aggregates and block of aggrephagy in Schwann cells leading to hypomyelination, axon loss and reduced regenerative capacity of axons, (3) altered turnover of synaptic vesicles, (4) destabilization of the postsynaptic complex of NMJs and altered turnover of AChR, precocious denervation, (5) dysfunctional mitophagy leading to accumulation of damaged mitochondria in skeletal muscle. Proteins so far identified to be relevant in the context of autophagy and disease in the peripheral neuromuscular complex as mentioned in the text are indicated close to the corresponding described location. Numbers in brackets indicate citation numbers. location MF, muscle fiber; MN, motoneuron; SC, Schwann cell.

toxic proteins in neurons (Figure 1). For example, deletion of the autolysosomal regulator, Ectopic P granules protein 5 homolog (Epg5) (44) led to hind limb paralysis with altered motor unit action potentials. Epg5 primarily helps in clearing protein aggregates, as loss of this molecule led to accumulation of p62 aggregates (45). Furthermore, optic nerve cut caused increased damage of retinal ganglion cells in the absence of autophagy (Atg4B$^{-/-}$) and treatment with the autophagy stimulator rapamycin reduced the denervation-induced cell death (17). Motor neuron injury augments autophagic activity, likely for mediating cell death of highly damaged motor neurons and removing proteins from damaged areas, so that recycled amino acids might be used for survival and nerve regeneration (46,47). In many pathophysiological conditions alteration in the levels of autophagy precede symptom onset. For example, decrease in autophagy levels have been shown to be early symptoms of retrograde motor neuron degeneration after spinal root avulsion (48).

Apart from Epg5's role in clearing protein aggregates and peripheral neuropathy, a series of studies has addressed the role of Fig4 in peripheral neuron diseases. Fig4 (syn. phosphatidylinositol 3,5-bisphosphate 5-phosphatase or Sac3) is part of the ternary PIKfyve-ArPIKfyve-Sac3 complex and converts phosphatidyl inositol-(3,5)-bisphosphate to phosphatidyl inositol-(3)-phosphate. Thereby it regulates several aspects of the endomembrane system, in particular, the protein flux from endosome to lysosome. Loss of Fig4 function is involved in many neurological diseases, including familial epilepsy, Yunis-Varòn syndrome, amyotrophic lateral sclerosis (ALS), and Charcot-Marie-Tooth disease (CMT). Recent work using transgenic mouse models identified differential effects of loss of Fig4 on motor neurons and myelinating Schwann cells (49). Indeed, conditional inactivation of Fig4 in motor neurons led to vacuolization and degeneration of motor axons, showing a cell autonomous role of the protein in these nerve cells. Interestingly, the axons were

also hypomyelinated, suggesting an aberrant axo-glial communication (49). Conditional loss of Fig4 in Schwann cells induced altered endolysosomal trafficking, block in autophagic progression, and accumulation of organelles and vesicles in these cells. This was accompanied by thin or absent myelin sheaths, progressive axonal loss, and reduced regenerative capacities of the peripheral nerve upon lesion (49). The findings on Schwann cells and the role of Fig4 on autophagy link to further studies on CMT models.

CMT, also called hereditary motor and sensory neuropathy (HMSN) or peroneal muscular atrophy, comprises a group of late onset peripheral neuropathies that result in muscle weakness and pain. Major forms of CMT are due to mutations or overexpression of the peripheral myelin protein 22 (PMP22) in myelinating Schwann cells. PMP22 is formed and folded in the ER. Misfolded PMP22 proteins can either undergo further cycles of refolding or, if unsuccessful, be exported from the ER into the cytosol and degraded in the proteasome via the ER-associated degradation (ERAD) process. However, in the disease state, unfolded PMP22 might be present at excessive levels that overwhelm the proteasomal machinery thus leading to its accumulation. Indeed, different lines of evidence suggest that PMP22 production and degradation need to be in fine balance. For example, wildtype PMP22 accumulates upon inhibition of the proteasome (50) and upon PMP22 overexpression (51). Furthermore, the mutant PMP22 Leu16Pro protein that is expressed in the Trembler CMT mouse model, is degraded slower than wildtype protein, and accumulates in large perinuclear protein clusters, called aggresomes (52). Notably, aggresomes appear to be also involved in a couple of neurological diseases, including Alzheimer's, Huntington's and Parkinson's Disease, ALS, and Age-related Macular Degeneration. Upon ubiquitination, aggresomes are recognized by histone deacetylase 6 (HDAC6), which further enhances protein accumulation (53). Coupling of aggresomes to dynein motor protein and acetylation of microtubules would allow the retrograde transport of the aggresomes towards the perinuclear region where eventually autophagosome formation and fusion with the lysosome occur (54). In this context, the HDAC6-interaction chaperone, p97/VCP (valosin-containing protein), regulates the disassembly of the HDAC6 complex and supports the autophagic degradation of the aggresome (53,55). Notably, p97/VCP is linked to a couple of aggresome-related diseases, including familial ALS (56), hereditary spastic paraplegia (57), inclusion body myopathy associated with Paget's disease and frontotemporal dementia (58), and CMT (59). Since aggresomes appear to be potentially toxic (60) and their removal involves autophagic degradation, a couple of studies have analyzed, if stimulation of autophagy could be beneficial in the context of aggresome-related diseases. Indeed, dietary restriction (61,62) and rapamycin (63) alleviated CMT phenotypes in mice. The interplay between unfolded protein stress and proteasomal versus autophagic decay is also critical in bortezomib-induced peripheral neuropathy. The proteasome inhibitor bortezomib has been an effective treatment option in multiple myeloma patients (64). However, peripheral neuropathy is a severe side effect of this treatment. Although different reasons underlying bortezomib-induced peripheral neuropathy have been discussed, the induction of ER stress and autophagy (65) as well as the formation of aggresomes (66) were reported upon bortezomib incubation in isolated Schwann cells.

In the context of aggresome clearance, ubiquitination of aggresome-contained target proteins is of great importance for stimulating selective autophagy via p62/SQSTM1. This function seems to be executed by different ubiquitin ligases. TRIM50 was found to interact with HDAC6 and p62/SQSTM1 (67) and to be modulated by HDAC6 (68). Another set of studies suggests that a complex of the heat-shock 22 kDa protein 8 (HspB8), the co-chaperone Bag3, the chaperone Hsc70, and the E3 ubiquitin ligase CHIP is critical (69) for aggresome degradation in a process termed chaperone mediated selective autophagy. In particular, HspB8 has been linked to peripheral neuropathies, including distal hereditary motor neuropathy type II (70), spinal bulbar muscular atrophy (SBMA) (71), and ALS (71-73). The involvement of HspB8 in peripheral neurodegeneration is supported by different observations. First, HspB8 is upregulated in mouse models and human spinal cord samples from ALS patients, and particularly in the ventral horn. Trehalose, an autophagic stimulator enhances HspB8 expression. Particularly, in SBMA the toxic aggregates of misfolded androgen receptors have been shown to be processed via HspB8 directing these aggregates to autophagic degradation by preventing the formation of p62 bodies (71). Pharmacological impairment of autophagy blocks HspB8 functionality (72,73). Additionally, investigation of the $HspB8^{K141E}$ mutation, which is present in distal hereditary motor neuropathy type II patients, showed reduced targeting of autophagosomes to the lysosome (70). Finally, another link between peripheral disease, protein aggregates and autophagy has been recently described. Indeed, mutation in dystonin, which is involved in maintaining cytoskeleton and membrane transport, causes a lethal form of hereditary sensory and autonomic neuropathy in humans (HSAN-VI). Loss of dystonin function leads to aggregates of p62 and reduces autophagic flux in sensory neurons which ultimately leads to sensory neuropathy (74). In summary, the present literature suggests that aggrephagy is a principal process involved in peripheral axonal disorders (Figure 1). Conversely, the current understanding on the roles of autophagy in skeletal muscle as a major target tissue of peripheral neurons point more towards regulation of turnover of ion channels and mitochondria. In the following we will introduce the present knowledge

on autophagy functions first at the NMJ and then at the sarcomeric region of skeletal muscle.

3.2. Autophagy for synaptic integrity and receptor turnover

Due to its large size, easy accessibility and eminent physiological role in triggering voluntary muscle contraction, the NMJ has been one of the most widely studied synapses across the animal kingdom. While initially many studies have looked at NMJs from a developmental perspective, the important role of these synapses in age-related muscle wasting (sarcopenia) has spurred interest on understanding the molecular pathways for maintaining NMJs as well (75,76). Here, we will focus on the role of the autophagic molecular machinery in maintaining NMJs in the post developmental phase. For the role of autophagy in synaptogenesis readers may refer to a recent review of Shen *et al.*, 2015. In vertebrates, the NMJ apparatus involves at least four cell types (1). While the NMJ core machinery consists of a one-to-one coupling of cholinergic alpha motor neuron and skeletal muscle fiber, myelinating and terminal Schwann cells are crucial for insulating the axon and the synaptic terminal. In addition, kranocytes (NMJ capping cells) (77) and sympathetic neurons (78,79) have been recently found in the immediate vicinity of NMJs, but their functions need further exploration. The postsynaptic region of the vertebrate neuromuscular complex is highly convoluted into junctional folds. While crests of these folds harbor acetylcholine receptors (AChRs), the troughs contain voltage gated sodium and potassium channels (80). From a physiological point of view, junctional folds are key to regulate the safety factor of neuromuscular transmission, which is presented as the ratio between the actual endplate potential and the threshold for generating a muscle action potential (81). The amount of acetylcholine released presynaptically and the density of AChRs present at the junctional folds are important determinants of the safety factor and linked to autophagy (Figure 1).

Autophagosomal degradation of synaptic vesicles has been described in Drosophila larval motor neurons. It was shown that this function is mediated via Rab26 being a direct effector of the autophagy related protein Atg16L1 (82). As recently reviewed (16), autophagy is also implicated in turnover of postsynaptic receptor proteins, such as $GABA_A$ receptors (83), NMDA receptors (84), and AChR (39) in mammals. In all three cases autophagic processing of receptors was strongly linked to models of disuse or lack of nutrients. In skeletal muscle, lysosomal inhibitors reduce AChR degradation rate (85,86). It was reported that AChRs undergo autophagic degradation that is regulated by the E3 ubiquitin ligase MuRF1 (39). Accordingly, AChR degradation was reduced in MuRF1-KO mice upon denervation (38) and starvation (39). The importance of autophagy for the maintenance of NMJ was also demonstrated in conditional knockout mice lacking the autophagy mediator ATG7 in skeletal muscle. Indeed, NMJs of these mice exhibited strongly altered morphology, AChR turnover and lacked co-alignment of the principal AChR clustering factor MuSK with AChR (87). NMJs in these muscles showed precocious denervation (87). Of course, it cannot be excluded that these phenotypes were due to indirect effects such as general alterations of endomembrane trafficking or mitochondrial stability. Indeed, mitochondrial function was also impaired in these mice (87).

As recently reviewed (75,76), NMJ dysfunction is increasingly recognized as a key factor of age-related progressive muscle loss and weakness (sarcopenia). The observation, that NMJ morphology and innervation status can be amended by physical exercise and caloric restriction (88-92), the latter being a classical way to induce autophagy, further augmented interest in this matter and spurred the search for NMJ-specific biomarkers of sarcopenia (93,94). Notably, a carboxy-terminal fragment (CAF) of the motor neuronal AChR clustering factor agrin has been identified a candidate as a marker of sarcopenia and NMJ remodeling in several studies (95-101).

In the light of selective autophagy, it is tempting to speculate on a revised role of various E3 ubiquitin ligases in directing proteins to autophagosomal pathways. In *C. elegans*, E3 ubiquitin ligases such as Mib1 ubiquitinate and promote degradation of SMN (survival of motor neuron protein) (102). 90% of spinal muscular atrophy results from the deletion of SMN gene. Similarly, LISTERIN (103) and RPM1 (104), have also been shown to be important for motorneuron survival in mouse and *C. elegans* models, however the role of these E3 ubiquitin ligases in selective autophagic degradation should also be considered for future investigation.

3.3. Autophagy and mitochondrial dysfunction in human pathologies

Autophagy appears to be critical under conditions of ER stress, overwhelmed proteasomal function and the removal of toxic protein aggregates. A key function of autophagy related to the peripheral neuromuscular apparatus is the maintenance of mitochondrial homeostasis (Figure 1). Mitochondrial quality control, which describes how to maintain functional mitochondria, is crucial for proper cellular and mitochondrial homeostasis. Autophagic clearance of damaged mitochondria, termed mitophagy, refers to an important part of mitochondrial quality control (105,106). Healthy mitochondria are required for energy supply of cells, while aged or damaged mitochondria release toxic reactive oxygen species and induce apoptosis (107). Accordingly, in health mitochondrial biogenesis and degradation are precisely balanced, and mutational disruption causes mostly neurodegenerative and heart

diseases, like inheritable Parkinson's disease (108,109). In the context of neuromuscular human pathologies, comprising motor neurons as well as skeletal muscle, mitochondrial dysfunction has been linked to disease, for example in the context of ALS (110), Friedreich Ataxia (111), and CMT (59), as well as in human muscular dystrophies, like Ullrich congenital muscular dystrophy (UCMD), Bethlem myopathy and congenital myosclerosis (112). Genetic studies linked inherited Parkinson's disease to mutations of mitophagy genes, Parkin (E3 ubiquitin ligase) and serine/threonine protein kinase PINK1 (phosphatase and tensin homolog (PTEN)-induced putative kinase protein 1) (113). In healthy mitochondria, the amino-terminus of PINK1 is entering the translocase of the inner mitochondrial membrane (TIM) in a membrane potential-dependent manner and is processed by the inner membrane rhomboid protease PARL, which cleaves within the transmembrane segment and generates an instable amino-terminal fragment of PINK1. The leftover carboxy-terminal part of cleaved PINK1 is translocated back into the cytosol and degraded by the ubiquitin-proteasome system (different views have been reported whether PINK1 is first processed by MPP or not) (114-116). Absence of the mitochondrial inner membrane potential stabilizes PINK1 on damaged mitochondria, labeling them for binding of Parkin, Parkin-mediated ubiquitination, and mitophagic elimination (117). The receptor for Parkin is Mitofusin 2. Mitofusins (Mfn 1 and 2) are mitochondrial outer-membrane fusion proteins and Parkin ubiquitination substrates (118). Mfn1 and Mfn2 in mouse hearts induces mitochondrial dysfunction and fragmentation that should stimulate mitophagic removal but instead results in proliferation of abnormal organelles (119). Mfn2 is phosphorylated by PINK1 and likely functions as receptor for Parkin. Parkin-mediated ubiquitination of mitochondrial proteins (including mitofusins) leads to degradation of damaged mitochondria by mitophagy (120,121). In skeletal muscle, both the absence as well as hyperactive autophagy appears to be detrimental. Indeed, upon autophagy-induction by starvation or activation of the FOXO3 axis, skeletal muscle mitochondria are heavily fragmented and exhibit unstable membrane potential (122). Moreover, vigorous activation of autophagy by genetic, dietary and pharmacological approaches restored myofiber survival and ameliorated the dystrophic phenotype of collagen 6 alpha 1 deficient mice (123). However, the same is true in mice lacking autophagy in a muscle-specific manner (87,124), suggesting that a fine balance and muscle status-dependent activation of autophagy is critical for the maintenance of a functional mitochondrial network in skeletal muscle.

4. CONCLUDING REMARKS

Impaired autophagy is a hallmark of several diseases of the central and peripheral nervous system. The peripheral neuromuscular transmission apparatus, that involves motor neurons, Schwann cells, and muscle fibers, is susceptible to dysfunctional autophagy in different manners. In these cell types, clearing of protein aggregates, mitochondria, and transmembrane receptors are the major targets of autophagy. Given that some transmembrane receptors are degraded via autophagy the role of this degradative pathway in the regulation of downstream signaling is worth studying. Finally, a growing body of literature indicates that the stimulation of autophagy by pharmacological or metabolic approaches might be a valid way to counteract certain forms of neuromuscular transmission defects.

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