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Review

Crosstalk between presynaptic trafficking and autophagy in Parkinson's disease

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ABSTRACT

Parkinson's disease (PD) is a debilitating neurodegenerative disorder that profoundly affects one's motor functions. The disease is characterized pathologically by denervation of dopaminergic (DAergic) nigrostriatal terminal and degeneration of DAergic neurons in the substantia nigra par compacta (SNpc); however, the precise molecular mechanism underlying disease pathogenesis remains poorly understood. Animal studies in both toxin-induced and genetic PD models suggest that presynaptic impairments may underlie the early stage of DA depletion and neurodegeneration (reviewed in [Schirinzi, T., et al. 2016](#)). Supporting this notion, human genetic studies and genomic analysis have identified an increasing number of PD risk variants that are associated with synaptic vesicle (SV) trafficking, regulation of synaptic function and autophagy/lysosomal system (Chang, D., et al. 2017, reviewed in Trinh, J. & Farrer, M. 2013; Singleton, A.B., et al. 2013). Although the precise mechanism for autophagy regulation in neurons is currently unclear, many studies demonstrate that autophagosomes form at the presynaptic terminal (Maday, S. & Holzbaur, E.L. 2014; Vanhauwaert, R., et al. 2017; reviewed in Yue, Z. 2007). Growing evidence has revealed overlapping genes involved in both SV recycling and autophagy, suggesting that the two membrane trafficking processes are inter-connected. Here we will review emergent evidence linking SV endocytic genes and autophagy genes at the presynaptic terminal. We will discuss their potential relevance to PD pathogenesis.

1. Introduction

1.1. Synaptic vesicle recycling

The presynaptic terminal is a highly specialized structure where SVs cluster in the proximity of the electron-dense active zone, and are prepared physically and biochemically for fusion and recycling with the action of a highly conserved group of molecules. Vesicle fusion is mainly triggered by calcium entering through voltage-gated calcium channels upon membrane depolarization. Calcium binds to SV-resident calcium sensors (the synaptotagmin family members) ([Brose et al., 1992](#)), and catalyzes the zippering of the four-helix molecular complex, known as the SNAREs, to mediate SV fusion to the plasma membrane. This process occurs in the time scale of several milliseconds. The SNARE complex is quickly dissociated by an ATPase, NSF, and restores the original protein conformation ready for the next fusion event ([Sollner et al., 1993](#)). SV protein cargos and lipid components are subjected to recycling, and in some cases, sorting, after the fusion event. Although the mode of SV recycling and the typical time scale are highly debated

(reviewed in [Granseth et al., 2007](#); [He and Wu, 2007](#); [Smith et al., 2008](#)), it is generally accepted that an adaptor protein (such as AP2) is required for SV cargo detection and clathrin recruitment to initiate the most classic form of endocytosis. Other molecules, such as endophilin, dynamin, synaptojanin1, auxilin, are involved at various stages of the endocytic process to mediate membrane deformation, fission and clathrin uncoating. Majority of the endocytosed SVs are reused after neurotransmitter refilling driven by the pH gradient, while a fraction of them are sorted in the endosome system to become new SVs.

1.2. Autophagy

Autophagy, which refers to “self-eating” from the Greek terms, describes the cellular process in which cytosolic components, including mainly long-lived proteins and organelles are transported to lysosome for degradation (reviewed in [Klionsky, 2007](#); [Nixon, 2013](#)). Macroautophagy is the most common form of autophagy subtype and will be simply referred to as “autophagy” in the following discussion unless otherwise noted. Microautophagy is characterized by direct

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invagination of the lysosomal membrane during sequestration of cytoplasmic contents and is the least understood phenomenon in mammalian cells. Chaperone-mediated autophagy, also known as CMA, features the most selective translocation of the cytosolic proteins to the lysosomal lumen via cytosolic chaperones, which are specifically recognized by receptors at the lysosomal membrane (Cuervo, 2010). All three types of autophagy coexist in neurons to maintain cellular homeostasis. They aid in the clearance of misfolded proteins, disease-prone aggregates and damaged organelles and play roles in neurodevelopment and plasticity (Yue, 2007; Wong and Cuervo, 2010). More than 30 autophagy-related (ATG) genes have been identified. Most of them are evolutionarily conserved; they are functionally distinct and involved in the synthesis and trafficking of autophagosomes, fusion between autophagosomes and lysosomes and final degradation in lysosomes. The roles of autophagy genes in neurons and regulation of neuronal autophagy are just beginning to be understood.

1.3. Crosstalk between endocytosis and autophagy

Recent findings have suggested crosstalk between mechanisms in regulating SV endocytosis and autophagy. For example, clathrin, which was known to coat the endocytic membranes during SV recycling, has been reported for its new cooperative roles with PI(4,5)P₂ in autophagic lysosome reformation (Rong et al., 2012). Additional evidence also implicated clathrin in the microautophagy pathway (Oku et al., 2017). Besides clathrin, membrane remodeling protein, endophilinA1, and its close binding partner, synaptojanin1, have also been proposed to regulate autophagosome biogenesis as well, which will be detailed in the following sections. Supporting this notion, human genetic studies and genomic analysis have identified an increasing number of PD risk variants that are associated with both SV trafficking and autophagy/lysosomal system (Chang et al., 2017, reviewed in Trinh and Farrer, 2013; Singleton et al., 2013). Although the precise mechanism for autophagy regulation in neurons is currently unclear, many studies demonstrate that autophagosomes form at the presynaptic terminal (Maday and Holzbaur, 2014; Vanhauwaert et al., 2017; reviewed in Yue, 2007), and are transported retrogradely via the assistance of a motor protein, dynein, for further degradation at the cell body (Cheng et al., 2015). Growing evidence has revealed overlapping genes involved in both SV recycling and autophagy, suggesting that the two membrane trafficking processes are inter-connected (Fig. 1).

2. LRRK2 regulates both autophagy and SV trafficking

Dominantly inherited mutations in *LRRK2/PARK8* are linked to both familial and sporadic cases of PD (reviewed in Mata et al., 2006; Paisán-Ruiz et al., 2013; Zimprich et al., 2004). LRRK2 is a large complex protein that contains multiple functional domains, including leucine-rich repeats (LRR), a Ras-of-Complex (Roc) GTPase domain, a C-terminal-of Roc (COR) domain, a tyrosine-kinase-like protein kinase domain and WD40 repeats. Majority of the PD mutations were found within the central Roc-COR tandem and the kinase domain. LRRK2 is considered the most frequent genetic cause for PD, and its role in autophagy and SV trafficking has been extensively studied in the recent years.

2.1. Wildtype LRRK2 in autophagy regulation

Several studies have shown that LRRK2 may regulate autophagy. In an earlier study using human embryonic kidney cell line (HEK293), LRRK2 knockdown was shown to increase autophagic flux (increased Atg8/LC3 lipidation) under starvation conditions (Alegre-Abarrategui et al., 2009), suggesting an inhibitory role of endogenous LRRK2. Using *LRRK2* knockout (*LRRK2*^{-/-}) mice, Jie Shen and colleagues carried out a series of investigations to characterize the physiological function of LRRK2 (Tong, Y., et al. 2010 and 2012; Giaime et al., 2017). Their

studies showed that, despite the lack of change in the brain, there is a biphasic change of autophagy in the kidney (Tong et al., 2012): loss of LRRK2 alone leads to an enhanced autophagic flux at 3–4 months; however, in 20-month old *LRRK2*^{-/-} mice, autophagy flux was reduced (Tong et al., 2010). Interestingly, the DAergic system of the *LRRK2*^{-/-} mice appeared normal up to 20 months of age (Tong et al., 2010), likely due to compensatory roles of LRRK1, the functional homologue of LRRK2. *LRRK2* and *LRRK1* double deleted (*LRRK*^{-/-}) mice displayed selective loss of DAergic neurons in the SNpc at 15 months, and an accelerated decline of autophagic clearance in the brain compared to WT mice (Giaime et al., 2017). These results suggest a compensatory role of LRRK1 in brain autophagic regulation, which explains the lack of obvious autophagy defects in *LRRK2*^{-/-} brain. Conditional deletion (or silencing) of LRRK2 at an older age using alternative strategies may be useful in determining the relevance of LRRK2 loss-of-function in brain autophagy and PD pathogenesis.

2.2. LRRK2 PD mutants in autophagy regulation

The G2019S mutation (GS) is the most common LRRK2 variant found in PD patients. Along with the R1441C/G/H (RC/RG/RH) mutations, they represent the most well studied PD mutations in LRRK2. The PD mutations cause enhanced kinase activity. A previous study showed that expression of the RC mutant in HEK293 cells led to impaired autophagic function evidenced by an elevation of large autophagic vacuoles and an increased level of p62 (Alegre-Abarrategui et al., 2009). BAC transgenic mice expressing human LRRK2 RG (hRG BAC) developed intra-axonal autophagic vacuoles in the nigral DAergic projecting axons, indicating disturbance in the autophagy/lysosomal pathway (Tagliaferro et al., 2015). The GS mutation, on the other hand, was shown to stimulate autophagy in differentiated neuroblastoma cells, and its impact on shortened neurite lengths could be reversed by RNAi-mediated knockdown of Atg8/LC3 or Atg7 (Plowey et al., 2008). However, using a LRRK2 pharmacological inhibitor, IN-1, one study showed that block of LRRK2 kinase activity stimulates autophagy in human neuroglioma cells (Manzoni et al., 2013). With recent evidence on the off-target effect of IN-1 (Luerman et al., 2014), this conclusion remains to be confirmed using more specific LRRK2 kinase inhibitors. In *C. elegans*, expressing either GS or RC mutant of LRRK2 inhibited autophagy (Saha et al., 2013). Despite the existing evidence implying an active role of LRRK2 in autophagy regulation, the results appeared rather conflicting. One study suggests that LRRK2 may activate the CaMKK-β/AMPK pathway and act as a modulator of lysosomal calcium and proton homeostasis to impact autophagy and cell survival (Gómez-Suaga and Hilfiker, 2012). This hypothesis has yet to be verified in other model systems. The exact role and the mechanism for LRRK2 in regulating autophagy thus remain largely unclear.

Clearance of mutant LRRK2 has been thought of as a protective mechanism during protein stress. Besides proteasome degradation (Ko et al., 2009), LRRK2 was also found to be a substrate of CMA and could be degraded in lysosomes (Orenstein et al., 2013). However, unlike typical CMA substrates, which competes for lysosomal association, the lysosomal LAMP-2A binding for LRRK2 exhibit dose-dependent increase upon addition of other CMA substrates. Such atypical interplay between LRRK2 and CMA could compromise the efficient degradation of other CMA substrates, such as α-synuclein (see below). LRRK2 G2019S mutant displayed further impaired degradation by CMA compared to WT proteins, and it also exerts greater inhibition over this pathway (Orenstein, S.J., et al. 2013). It is possible that the impaired CMA leads to an enhanced compensatory macroautophagy function (Plowey et al., 2008), however, whether and how such compensation lead to additional toxic effects in PD pathogenesis remains to be understood.

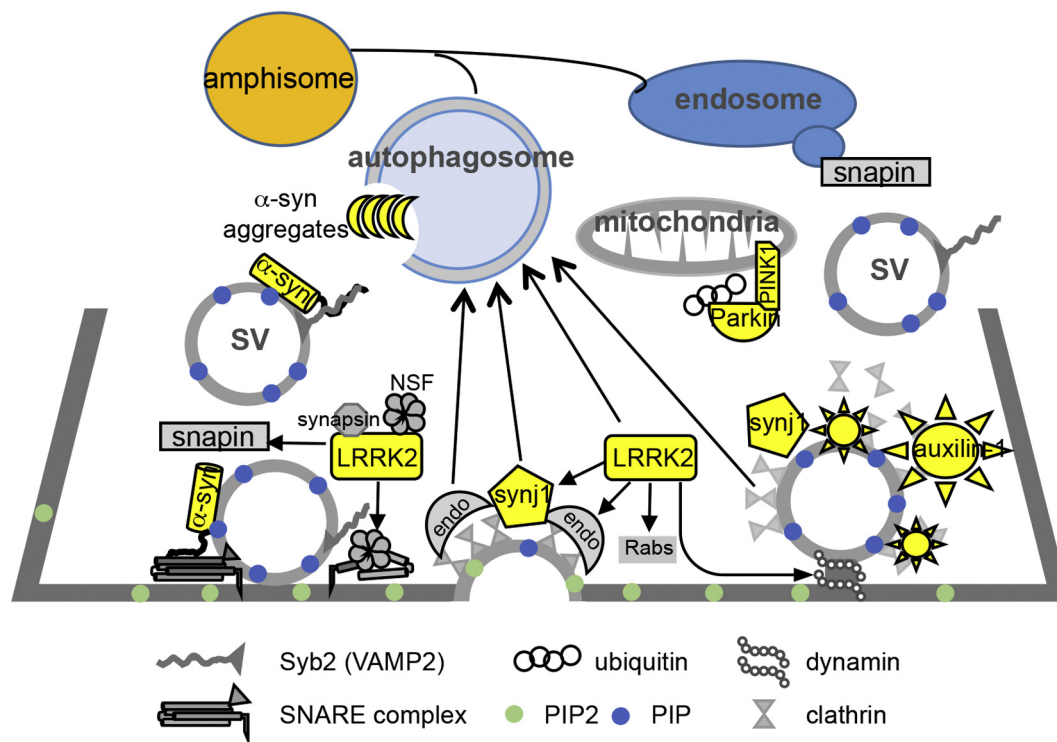


Fig. 1. PD genes in regulating both autophagy and SV trafficking.

A cartoon illustrates several PARK genes (highlighted in yellow) at the presynaptic terminal and their roles in regulating SV exocytosis/endocytosis as well as their proposed connections to autophagy. As a promiscuous kinase, LRRK2 has been shown to regulate SV recycling via phosphorylating several synaptic targets (solid arrow heads), many of which are known to be involved in autophagosome formation (open arrow heads) as well.

2.3. LRRK2 interaction with synaptic proteins

Extensive studies have also suggested a role of LRRK2 in regulating SV recycling. LRRK2 was found to be present in the SV fraction and associate with SV membrane proteins, such as synaptophysin, VAMP2, and synapsin I. It was also shown to interact with cytosolic proteins such as NSF, MUNC18-1, dynamin-1, Rab5b and Rab7L1, and presynaptic membrane proteins, such as syntaxin 1A, which participate in regulating SV fusion and recycling (Piccoli et al., 2014; Cinaru et al., 2014; Shin et al., 2008; Yun et al., 2015; MacLeod et al., 2013). Besides, LRRK2 coimmunoprecipitates with actin and tubulin, further supporting LRRK2 as part of a complex synaptic protein network involved in neurotransmission and synaptic remodeling (Piccoli et al., 2014; Cinaru et al., 2014; Plowey et al., 2008).

Most PD-linked mutations (including the G2019S mutation in the LRRK2 kinase domain and the R1441C/G/H and Y1699C mutations in the ROC-GTPase domain) result in enhanced kinase activity (West et al., 2005; Li et al., 2010; Steger et al., 2016; Islam et al., 2016), and therefore the “gain-of-toxic-function” has been proposed as a potential pathogenic mechanism for LRRK2-mediated pathogenesis. The theory has led to identification of numerous targets of the LRRK2 kinase, many of which are synaptic proteins (Fig. 1), such as Snapin (Yun et al., 2013), NSF (Belluzzi et al., 2016), Rab5b (Shin et al., 2008; Yun et al., 2015), endophilin (Matta et al., 2012; Arranz et al., 2015) and synaptojanin1 (Islam et al., 2016; Pan et al., 2017). While many of these potential substrates remain to be validated in vivo, LRRK2 phosphorylation of synaptojanin1 and endophilinA were confirmed in flies expressing human LRRK2 mutants (Matta et al., 2012; Islam et al., 2016). A recent investigation employed a combination of phosphoproteomics, mouse genetics, and pharmacological LRRK2 inhibitors and identified a subset of Rab GTPases, including Rab8, Rab10 and Rab12 as bona-fide LRRK2 substrates (Steger et al., 2016). The pathogenic implication, however, remains to be understood. There are at least 60 Rab genes in

the human genome and they function as regulators for a multitude of intracellular membrane trafficking pathways by recruiting effector molecules to their GTP-bound forms (reviewed by Zerial and McBride, 2001). It is likely that phosphorylation of the Rabs results in interference with membrane reorganization during synaptic transmission as well as during autophagosome trafficking and recycling.

2.4. LRRK2 regulation in SV trafficking

In parallel to the biochemical information on the altered protein network at the presynaptic terminal, LRRK2 has been shown in various experimental settings to modulate synaptic transmission and SV recycling.

2.4.1. LRRK2 and SV endocytosis

An early study showed that in cultured hippocampal neurons, expressing either wildtype (WT), GS or RC mutants of LRRK2 slowed SV endocytosis, while knocking down LRRK2 by RNAi led to a more significant impairment in SV endocytosis (Shin et al., 2008). This study, however, contradicts with a recent study using the same optical probe (pHluorin) that failed to observe changes in SV endocytosis in cultured hippocampal neurons from LRRK2^{-/-} or LRRK1 and LRRK2 double deleted mice (Maas et al., 2017). Interestingly, LRRK2^{-/-} striatal neurons displayed impaired SV endocytosis (Arranz et al., 2015). Although it is unclear whether such alteration occurs in vivo or is associated with the role of LRRK2 in PD pathogenesis, it suggests that LRRK2 may regulate SV recycling in a cell type specific manner. Supporting this notion, our recent study showed that hyperactivity of the LRRK2 GS mutation specifically slowed SV endocytosis in cultured midbrain neurons (containing DAergic neurons), but not cortical neurons (Pan et al., 2017). In our study, we have also observed a modest slowing of SV endocytosis in LRRK2^{-/-} cortical neurons while sparing the midbrain neurons. In *drosophila* neuromuscular junction (NMJ),

either deleting the *LRRK* gene or expressing human *LRRK2 GS* resulted in the slowing of SV endocytosis (Matta et al., 2012). The authors proposed that such bi-directional regulation of SV endocytosis by *LRRK2* might be due to the unique biophysical role endophilinA (endoA) as a *LRRK2* substrate (Ambroso et al., 2014). It is possible that both endoA and its close binding partner, synaptojanin1, are compromised by *LRRK2* phosphorylation (Pan et al., 2017; Islam et al., 2016), leading to a failed protein network for supporting efficient SV recycling. Nevertheless, the differential regulation of this network in distinct cell types awaits further investigation.

2.4.2. *LRRK2* and SV exocytosis

Removing endogenous *LRRK2* has not been reported to alter SV exocytosis during continuous synaptic activity. However, in primary cortical neurons prepared from *hLRRK2 GS* BAC mice, SV exocytosis was substantially enhanced, which can be reversed by a *LRRK2* kinase inhibitor, GSK2578215A (Belluzzi et al., 2016). Similar kinase activity-dependent facilitation of SV exocytosis was also observed for cortical neurons derived from *mLRRK2 GS* BAC mice (Pan et al., 2017). In contrast, exocytosis of midbrain neurons was not affected by the *GS* mutation. The enhanced exocytosis in *GS* expressing cortical neurons could be due to phosphorylation of NSF, which results in enhanced dissociation of the SNARE complex (Belluzzi et al., 2016). In the brief time scale when exocytosis was examined in the above studies, calcium homeostasis is believed to play an essential role in modulating SV fusion. Recent evidence for a direct regulatory role of *LRRK2* on Cav2.1 channel (Bedford et al., 2016) calls for a more detailed analysis in this arena. As calcium was shown to regulate autophagy (reviewed in Bootman et al., 2017), it is unclear if the change of calcium levels in response to action potential also alters autophagy pathway at axon terminal.

2.4.3. *LRRK2* and synaptic transmission

In rat cortical neurons, Plowey et al. showed that *LRRK2 GS* mutant enhances glutamatergic synapse activity by increasing the number of contacts (Plowey et al., 2014). A recent *in vivo* study using *hLRRK2 GS* KI mice, however, found no difference in the number of glutamatergic synapse in the striatum, despite the overly active glutamatergic transmission observed at postnatal day 21 (Matikainen-Ankney et al., 2016). The authors also found that the effect is kinase activity dependent and originates from the presynaptic site, consistent with the *GS* mutation-induced enhancement in exocytosis in cultured cortical neurons (Belluzzi et al., 2016; Pan et al., 2017). In aged (8–12 months) *mLRRK2 GS* BAC mice, the enhanced basal level of excitatory synaptic transmission was found in the hippocampal Schaffer collateral–CA1 synapse. However, the change did not appear to be from the presynaptic terminal (Sweet et al., 2015). Although there are apparent differences in the age and the brain region examined, it is certainly possible that *LRRK2* modulates synaptic transmission from both pre- and postsynaptic sites.

DAergic transmission has been independently evaluated by cyclic voltammetry as conventional electrical recording cannot access the G-protein coupled DA receptors. Using a complement of *LRRK2* kinase inhibitors, DA release was not altered at various stimulations patterns (Qin et al., 2017). In 12-month old *mLRRK2 GS* BAC mice, we previously reported decreased release of DA in response to single as well as prolonged stimulations (Li et al., 2010). Similar impairment was found in two other different *hLRRK2 GS* transgenic lines (Liu et al., 2015; Chou et al., 2014). However, in *hLRRK2 RC* BAC mice, evoked DA release was not altered at 6–8 weeks of age (Sanchez et al., 2014), despite their abnormal DA-dependent motor functions starting at 6 months (Li et al., 2009). In a recent study in *hLRRK2 WT, GS* and *RC* BAC transgenic rats, evoked DA release was also found reduced when rats aged to 18–22 months (Sloan et al., 2016). These results from multiple groups and the examination of different animal models consistently suggest an age-dependent inhibitory role of *LRRK2 PD* mutants in DAergic transmission. The mechanism of reduced DAergic transmission remains

unknown. Whether it is due partially to altered SV exocytosis and endocytosis is yet to be demonstrated in the *in vivo* model. Other impairments, such as a decreased capability for neurotransmitter refilling in aged DA neurons (Liu et al., 2015), may also account for the *LRRK2* mutation-induced age-dependent impairment in DAergic transmission.

2.5. The role of *LRRK2* in autophagy/SV trafficking crosstalk

Despite substantial evidence demonstrating separate roles of *LRRK2* in SV trafficking and in autophagy regulation, it remains unclear whether these are two independent pathways of *LRRK2* are interconnected. The challenge lies in the lack of knowledge and strategy to study autophagy in neurons from the central nervous system where SV trafficking is well characterized. Several elegant works reported autophagosome formation at the presynaptic terminal (Maday and Holzbaur, 2014; Vanhauwaert et al., 2017), however, most of these works are based on mammalian periphery neurons (dorsal root ganglion/DRG neurons) or non-mammalian synapses (*Drosophila* neuromuscular junction/NMJ). Autophagosome formation in nerve terminals of central neurons has not been reported or consistently observed using conventional autophagy induction strategies, such as starvation or inhibition of the mTOR pathway. It is likely that autophagy is not a robust and frequent cellular event at central synapses and cannot be easily captured; or, perhaps autophagosome formation in central synapses require unconventional induction strategies, which are yet to be identified. Nevertheless, it remains possible that *LRRK2* uses similar molecular signaling complexes in mediating both SV trafficking and autophagosome formation at nerve terminals. Careful analyses in DRG neurons or *Drosophila* NMJ, similar to what has been demonstrated for endophilinA (detailed below), may help elucidate this potential crosstalk.

3. Presynaptic protein α -synuclein interacts with autophagy and SV membrane

A-Synuclein (α -syn), encoded by the *SNCA/PARK1, 4* genes, is the key protein component of Lewy bodies present in the parkinsonian brain. It is a presynaptic terminal-enriched small (14 kDa) protein containing three functional domains: the highly conserved N-terminal domain, which can form amphipathic α helices for lipid interaction (Davidson et al., 1998), the central hydrophobic NAC domain, which is involved in formation of β -sheet rich amyloid-like fibrils (Giasson et al., 2001), and an unfolded C-terminal tail, which is known to bind VAMP2/synaptobrevin-2. All PD-linked disease mutations, A30P, E46K, H50Q, G51D and A53T (Polymeropoulos et al., 1997; Kruger et al., 1998; Zarranz et al., 2004; Proukakis et al., 2013; Appel-Cresswell et al., 2013; Lesage et al., 2013), lie in the amphipathic region, suggesting a role of α -syn/lipid interaction in PD pathogenesis. Besides genetic variants, α -syn overexpression (gene duplication or triplication) also leads to familial early-onset severe forms of PD (Singleton et al., 2003; Chartier-Harlin et al., 2004).

3.1. α -synuclein and autophagy

Three degradation pathways, including CMA, autophagy and Ub-proteasome were shown to regulate the turnover of α -syn. A-syn contains a pentapeptide motif (KFERQ), which can be recognized by the chaperone protein HSC70, essential for CMA-mediated degradation (Cuervo et al., 2004). HSC-70 bound α -syn can then be transported into the lysosome via interaction with LAMP-2A. Mutant forms of α -syn (A30P, A53T) can bind much more strongly to LAMP-2A, and therefore failed to be transported across the membrane (Martinez-Vicente et al., 2008). Similar to *LRRK2* and mutant *LRRK2*, which were discussed above, such interference with normal function of CMA translocation complex reduces degradation of other CMA substrates, including damaged and misfolded proteins, resulting in cytotoxicity. A-syn can be

modified by oxidized dopamine, creating DA- α -syn, which displays a phenotype closely resembling that of the mutant α -syn. In various experimental conditions, CMA defect was observed when expressing DA- α -syn (Martinez-Vicente et al., 2008). Whether such α -syn-mediated toxicity underlies the pathogenic mechanism of PD remains to be tested particularly in vivo.

Besides CMA, current evidence also demonstrated a role of α -syn in interfering with macroautophagy or autophagy. Although mutant A30P and A53T α -syn blocks CMA (Cuervo et al., 2004), other studies found that expressing hA53T mutant α -syn enhanced autophagosome formation, likely due to a higher demand to remove α -syn proteins (Stefanis et al., 2001; Yu et al., 2009). In contrast, in mammalian cell lines, expressing hE46K mutant impairs autophagy induction. In cultured cells or neurons treated with preformed α -syn fibrils (PFFs), α -syn inclusions/aggregates, once formed, were shown to be refractory to degradation by autophagy due to reduced autophagosome clearance (Tanik et al., 2013). The exact mechanism for α -syn-mediated disruption of autophagy, however, remains controversial. Results obtained from mammalian cells and in transgenic mice suggest that overexpression of α -syn could compromise autophagy via Rab1a inhibition, accompanied by mislocalization of the autophagy protein, Atg9 (Winslow et al., 2010); and that overexpression of the E46K mutant could inhibit autophagy via deregulate the JNK1-bcl-2 pathway, but not the mTOR pathway (Yan et al., 2014). Whether the same regulation applies to other α -syn mutations is yet to be elucidated. As lipid interaction with α -syn might be essential for revealing its pathogenic role, more investigation of the impact of lipid metabolism on autophagy regulation is needed. Earlier studies suggested that α -syn inhibits phospholipase D (PLD) (Jenco et al., 1998; Ahn et al., 2002), a pivotal enzyme that regulates a multitude of phospholipid species and membrane trafficking pathways (reviewed in Cazzoli et al., 2006). Whether such alteration affects autophagosome formation remains unknown and awaits further investigation.

3.2. α -synuclein in SV trafficking

In the presynaptic terminal, it is believed that α -syn exists in the equilibrium of two forms: a native unfolded monomeric form and a SV membrane bound α -helical form. Evidence has shown that the membrane bound form is physiologically relevant and may form higher-order multimers to promote SNARE complex formation during SV exocytosis via interaction with VAMP2 (Burre et al., 2010; Diao et al., 2013; Burre et al., 2013) (Fig. 1). Surprisingly, deletion of α -syn, or even both α - and β -syn, had led to only minor impairments on glutamatergic transmission, which were observed during prolonged stimulation (Murphy et al., 2000; Cabin et al., 2002; Chandra et al., 2004). The nigral DAergic system, however, appears to be affected, which is represented by increased release of DA with paired or continuous stimuli in α -syn null mice (Abeliovich et al., 2000; Yavich et al., 2004; Senior et al., 2008). The enhanced release was shown to be a result of increased rate of SV refilling due to loss of α -syn (Yavich et al., 2004). However, it is somewhat at odds with the findings in hippocampal neurons, where the mobilization of the reserve pool of SVs is affected (Murphy et al., 2000). Introducing WT or mutant forms of α -syn has resulted in conflicting results. Transgenic mice expressing hWT α -syn impairs SV exocytosis in hippocampal neurons (Yavich et al., 2004), which is consistent with the findings in rat midbrain DAergic neurons (Nemani et al., 2010; Gaugler et al., 2012) or neuroendocrine cells (Larsen et al., 2006) overexpressing α -syn. Interestingly, while expressing A53T or E46K mutant of α -syn results in a similar inhibitory effect in SV exocytosis, A30P mutation failed to affect the rate of exocytosis (Nemani et al., 2010). Although it may seem plausible considering that only the A30P (not A53T or E46K) mutant protein exhibits significantly reduced membrane binding (Jo et al., 2002; Bussell Jr. and Eliezer, 2004; Bodner et al., 2010), it suggests an intriguing possibility that different mutations of α -syn work through distinct cellular

pathways in PD pathogenesis. More studies are required to understand how α -syn and its mutants interact with SV membranes in various neuronal types and modulate their dynamics in response to synaptic activities. These studies may ultimately lead to a better understanding of how α -syn/lipid interaction intersects SV recycling and autophagosome biogenesis.

4. EndophilinA: a junction of SV endocytosis and autophagy

Mammalian endophilinA (endoA) contains a family of three homologous genes - endoA1 (encoded by *SH3GL2*), endoA2 (encoded by the *SH3GL1*) and endoA3 (encoded by *SH3GL3*), among which, endoA1 is the only brain-specific isoform. EndoA is a member of the BAR protein family involved in membrane deformation, and its SH3 domain in the carboxyl terminal is capable of recruiting dynamin and synaptojanin1 (Fig. 1). The role of endoA1 in neuronal SV recycling was described 20 years ago (Ringstad et al., 1997). Following studies have demonstrated the impaired SV endocytosis in multiple animal models when two or more endoA isoforms are disrupted (Guichet et al., 2002; Verstreken et al., 2002; Schuske et al., 2003; Milosevic et al., 2011). It was not until recently that endoA was shown as a direct substrate of LRRK2 kinase at the NMJ of *Drosophila* (Matta et al., 2012). The authors have also found that the slowed SV endocytosis in *LRRK* mutant flies could be reversed by heterozygous mutation of the *Drosophila* endoA gene (Arranz et al., 2015). Although the in vivo evidence from a mammalian model is still lacking, this work represents the first evidence that endoA might be involved in PD pathogenesis via interaction with *LRRK2*. More interestingly, *SH3GL2* is now revealed in a meta-analysis as a significant risk gene for sporadic PD patients (Chang et al., 2017). The current research progress has brought a growing interest in understanding the role of endoA in DAergic neuron degeneration.

EndoA triple knockout (TKO) mice are lethal shortly after birth. Mice with endoA deficiency exhibit age-dependent motor impairments and progressive ataxia (Murdoch et al., 2016) reminiscent of neurodegenerative phenotypes in endoA1 and endoA2 double deleted (DKO) mice (Milosevic et al., 2011). Transcriptional analysis for the DKO and TKO hippocampus revealed the deregulation of autophagy and the Ub-proteasome system (Murdoch et al., 2016). Consistent with this idea, Soukup et al. showed that *Drosophila* endoA facilitates autophagosome formation at the presynaptic terminal by formation of highly curved membranes upon phosphorylation by LRRK. The curved membranes act as docking stations for recruitment of additional Atg proteins necessary for autophagosome maturation (Soukup S.F., et al. 2016). Although endoA-assisted membrane deformation is also required for SV endocytosis (Bai et al., 2010), and LRRK phosphorylation of endoA also modulates SV recycling (Matta et al., 2012), the role of endoA in autophagy regulation may be independent of its action in SV endocytosis (Soukup S.F., et al. 2016). The most compelling evidence is that LRRK phosphomimetic and phosphodead mutant of endoA exhibited opposite roles in autophagosome formation, but similar inhibitory roles in SV endocytosis.

The results presented in the series of work by Verstreken and colleagues are extremely exciting. It is also the first time that autophagy and SV endocytosis, the two presumably independent membrane trafficking processes, were examined at the presynaptic terminal as a potential cooperation via a common signaling pathway involving endoA. There are, however, several questions remain to be addressed. For example, how does LRRK phosphorylated endoA differentiate its designated role in SV endocytosis versus autophagy? Does endoA regulate SV trafficking and autophagy in a comparable way as in mammalian synapses? A major discrepancy between the fly NMJ and the mammalian central neuron synapse is that starvation (amino acid deprivation) does not induce autophagosome formation in cultured mouse hippocampal neurons (Maday and Holzbaur, 2016) as does for fly NMJ (Vanhauwaert et al., 2017; Soukup S.F., et al. 2016; Murdoch et al., 2016). In addition, there has been no report to date showing synaptic

activity-induced autophagosome formation in central neurons as does for fly NMJ, despite the observation that autophagosomes preferentially form at the presynaptic terminals of cultured dorsal root ganglion (DRG) and hippocampal neurons at basal conditions (Maday and Holzbaur, 2016).

5. Synaptojanin1 and auxilin in SV endocytosis and autophagy

Mutations in *SYNJ1* and *DNAJC6* were identified in the recent years in families with juvenile atypical Parkinsonism and are known as *PARK20* and *PARK19*, respectively (Krebs et al., 2013; Quadri et al., 2013; Olgiati et al., 2014; Kirola et al., 2016; Taghavi et al., 2017; Edvardson et al., 2012; Koroğlu et al., 2013). Patients carrying these mutations show similar early-onset symptoms and are often accompanied by seizure and other pyramidal dysfunctions. Synaptojanin1 (*synj1*) and auxilin are known to be close partners involved in clathrin coat removal after SV endocytosis (Fig. 1). Mice with *synj1* or auxilin deletion die shortly after birth, and their synapses exhibit accumulation of clathrin-coated vesicles (CCVs) (Cremona et al., 1999; Yim et al., 2010).

Synj1 is an essential inositol phosphatase enriched in the presynaptic terminal and auxilin is less characterized compared to *synj1*. *Synj1* is a 145-kDa protein containing three functional domains: the SAC1-like domain, which hydrolyzes inositol monophosphates, such as PI3P and PI4P; the 5'-phosphatase domain, which hydrolyzes PI(4,5)P₂ at the 5' position to produce PI4P; and the highly variable C-terminal proline-rich domain, which can be recruited by endoA during SV endocytosis. All three domains are involved in regulating SV recycling at various stages of synaptic activity (Mani et al., 2007). Interestingly, the disease related mutations are only present in the two phosphatase domains, suggesting an involvement of lipid deregulation in *SYNJ1*-mediated pathogenesis. Transgenic mice expressing disease mutation R258Q in the SAC1-like domain (*RQ KI* mice) recapitulated human symptoms and displayed dystrophic changes in the nigral DAergic terminals (Cao et al., 2017). In addition to an increased number of CCVs and reduced SV density in the dorsal striatum, onion-like membrane structures were observed in nigral DAergic terminals and striatal neurons, reminiscent of a burdened autophagy system in the absence of the essential autophagy genes (Komatsu et al., 2007; Nishiyama et al., 2007). In line with this observation, a recent study showed that the SAC1-like domain of *synj1* is required for autophagosome maturation at presynaptic terminals of fly NMJ (Vanhauwaert et al., 2017). The missense mutation in the SAC1-like domain (Krebs et al., 2013) impairs the recruitment of Atg18a/WIPI2 to further interrupt Atg8/LC3 lipidation. The evidence of *synj1*-mediated autophagy in mammalian central synapses is yet to be demonstrated. Interestingly, the role for clathrin and PI(4,5)P₂ in regulating autophagic lysosome reformation (ALR) was reported (Rong et al., 2012), and whether *synj1* is involved in ALR in neurons by regulating PI(4,5)P₂ remains unclear.

Similar to fly endoA, which is regulated by LRRK in NMJ, mammalian *synj1* was shown to be phosphorylated by LRRK2 in the proline-rich domain and the phosphorylation may comprise in part *synj1* endocytic function (Pan et al., 2017; Islam et al., 2016). *SYNJ1* haploinsufficiency leads to slowed SV endocytosis in midbrain neurons, reminiscent of those from *mLRRK2* GS BAC mice. Combining *mLRRK2* GS and *SYNJ1*^{+/-} mutations did not further impair SV endocytosis but reduced exocytosis during continuous stimulation, indicating a complex genetic interaction between *SYNJ1* and *LRRK2* in regulating SV trafficking. Given the recent advances in identifying the interplay among LRRK2 (LRRK), endoA and *synj1*, it is possible that the three molecules form a tripartite signaling complex in regulating synaptic membrane trafficking and autophagy, both of which may go awry in the pathogenesis of PD.

6. Autophagy proteins regulate SV recycling

Autophagy is a highly conserved cellular process across species. Deletion of essential autophagy gene *ATG5* or *ATG7* in brain leads to autophagy failure, accumulation of ubiquitinated proteins, neurodegeneration and mortality soon after birth (Kuma, et al., and Komatsu et al., 2006), suggesting that autophagy is neuroprotective by constant removal of cellular waste. Conditional deletion of *ATG7* in DAergic neurons results in enlarged, dystrophic DAergic terminals, age-dependent accumulation of α -syn, progressive loss of DAergic neurons and motor function deficits in mice (Friedman et al., 2012). Interestingly, a separate study showed that loss of Atg7 in DAergic neurons also leads to enhanced DA release as well as faster recovery after SV depletion (Torres and Sulzer, 2012). In contrast, inhibition of mTOR by rapamycin, which induces formation of autophagosomes in the presynaptic terminals of DAergic neurons, leads to decreased dopamine release (Hernandez et al., 2012). Whether such modulation in DA transmission is mediated by Atg7 and occurs in other parts of the brain is yet to be demonstrated in greater detail.

There is additional evidence suggesting that interference with the autophagy pathway could also modulate synaptic transmission. For example, Snapin is a SNAP-associated protein that was initially identified as a SNAP25 binding protein (Chheda et al., 2001). Its roles in mediating late endosomal trafficking (Cai et al., 2010; Kim et al., 2012), lysosomal acidification and autophagosome maturation (Shi et al., 2017) have recently been characterized. Snapin may form complex with dynein upon autophagosome fusion with late endosomes in distal axons, and thereby facilitate the retrograde transport of autophagic clearance at the soma (Cheng et al., 2015). Disruption of snapin or snapin-dynein interaction results in accumulation of autophagosomes in distal axons and synaptic terminals, indicating its involvement in autophagy clearance. Interestingly, *SNAPIN*^{-/-} cortical neurons also displayed reduced glutamatergic transmission (Pan et al., 2009) and impaired SV exocytosis (Di Giovanni and Sheng, 2015). In addition, snapin may regulate the SV pool size by interacting with dynein and facilitating the SV trafficking to the endosomal pathway (Di Giovanni and Sheng, 2015). Such regulation may represent an additional mechanism for crosstalk between SV trafficking and autophagy. SNAP29 is known as one of the three SNARE proteins besides syntaxin17 and VAMP8, in mediating fusion between autophagosomes and lysosomes in autophagy maturation step (Itakura et al., 2012; Takáts et al., 2013). Our early studies have found that SNAP29 could also act as a clamp for NSF-mediated SNARE complex dissociation at the presynaptic terminal and thereby inhibit continuous exocytosis at low frequencies (Su et al., 2001; Pan et al., 2005). The potential interplay between the two SNAP-29 regulated events remains unclear. An emergent school of thoughts propose that autophagy proteins are present at presynaptic terminals for degradation and turnover of presynaptic components, such as active zone proteins and SVs (Lüningschrör et al., 2017). The small GTPase, Rab26, has been implicated in this mechanism as it colocalizes with Atg16L1, LC3B and Rab33B (Binotti et al., 2015) and specifically directs synaptic vesicles to preautophagosomal structures (Lüningschrör et al., 2017). Although the current evidence of cross talk between autophagy and SV trafficking is rather limited, it has already begun to emerge as an exciting field of research for further elucidation of the interaction of the two membrane trafficking processes at presynaptic terminals.

7. Conclusion and outlook

The emerging evidence suggests extensive interaction between SV trafficking and autophagy pathways. Importantly several PD associated genes were found to play roles in regulating both SV trafficking and autophagy (Fig. 1). In support of above notion, other PD related genes such as *SYT11* (Pihlström et al., 2013; Wang et al., 2016; Bento et al., 2016; Wang et al., 2018), *TMEM230* (Deng et al., 2016; Kim et al.,

2017) and VPS35 (Vilariño-Güell et al., 2011; Zimprich et al., 2011; Inoshita et al., 2017) are also implicated in these two pathways. While the existing evidence shows that SV trafficking and autophagy are regulated by an overlapping set of genes, how exactly these overlapping genes switch for regulation of different pathways has yet to be elucidated. The challenge facing the field is lack of a reliable readout system for autophagy in the presynaptic terminals and the complexity in autophagy regulation at the mammalian central nerve terminals. Further studies are required to address these important and exciting questions. Gaining insight into the mechanism for the crosstalk between SV trafficking and autophagy may assist to reveal novel therapeutic targets for PD.

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