LRRK2, alpha synuclein, and tau: partners in crime or unfortunate bystanders?

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Perspectives

- LRRK2, aSyn and tau are associated with familial and sporadic forms of PD
- It is unclear whether LRRK2 interacts with and/or phosphorylates aSyn and tau
- A greater understanding of the connection between LRRK2, aSyn and tau may lead to the development of novel therapeutic strategies for PD

Abstract

The identification of genetic forms of Parkinson's disease (PD) has tremendously expanded our understanding of the players and mechanisms involved. Mutations in the genes encoding for alpha-synuclein (aSyn), LRRK2, and tau, have been associated with familial and sporadic forms of the disease. aSyn is the major component of Lewy bodies and Lewy neurites, which are pathognomonic protein inclusions in PD. Hyperphosphorylated tau protein accumulates in neurofibrillary tangles in the brains of Alzheimer's disease patients, but are also seen in the brains of PD patients. LRRK2 is a complex multi-domain protein with kinase and GTPase enzymatic activity. Since aSyn and tau are phosphoproteins, we review the possible interplay between the three proteins. Understanding the interplay between LRRK2, aSyn and tau is extremely important, as this may enable the identification of novel targets and pathways for therapeutic intervention.

Introduction

Parkinson's disease (PD) is a complex neurodegenerative disorder clinically known for typical motor features that include muscular rigidity, resting tremor, bradykinesia, and postural instability. In addition, a variety of non-motor features are also present, and develop progressively, prior and after the onset of motor features [1]. Lewy bodies (LBs) and Lewy neurites (LNs) are pathognomonic protein inclusions that accumulate in the brains of PD patients in a progressive manner and, usually, ascending from the brain stem into cortical areas [2–4]. The protein alpha-synuclein (aSyn) (Figure 1) is the major component of Lewy pathology [5,6].

While the vast majority of PD cases are sporadic (~90%), a subset is associated with genetic alterations [7]. Mutations in the genes encoding for aSyn (*SNCA*) and leucine rich repeat kinase 2 (*LRRK2*) (Figure 2) are associated with autosomal dominant forms of PD, and additional genetic variants are also associated with sporadic cases, as risk factors [8]. A growing number of other genes has also been linked to familial and sporadic forms of PD [9] and, together, are providing important insight into the complex molecular underpinnings of the disease.

Tau (Figure 3) is typically known as a microtubule binding protein that accumulates in neurofibrillary tangles in the brains of patients with Alzheimer's disease (AD) and other neurodegenerative diseases. In addition, mutations in the gene encoding for tau (*MAPT*) are associated with familial forms of frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) [10], but are also associated with sporadic forms of various disorders, including PD [11].

Neuropathological studies in brains of *LRRK2*-mutation carriers suggests significant variability, including typical LB pathology, but also ubiquitin, tau and/or TDP-43-positive inclusions [12].

The molecular interplay between different genes and proteins associated with PD is still unclear, but novel ideas are emerging as research advances. In particular, it is expected that a detailed understanding of the molecular mechanisms involved will enable the development not only of novel therapeutic strategies, but also of disease progression markers that are essential for following future clinical trials.

LRRK2: a complex protein whose function remains unclear

LRRK2 is a very large and complex protein, mainly characterized by its RocCOR-GTPase, and serine/threonine kinase domains, that confer specific enzymatic activities to the protein (Figure 2) [13–16].

The overexpression of LRRK2 leads to cytotoxicity that is GTPase and kinase-dependent, and PD-associated mutations cluster within the GTPase and kinase-domains, suggesting altered signaling that may have pathological implications (Figure 4) [13,14].

In vitro, LRRK2 can undergo autophosphorylation, but the GTPase activity of LRRK2 is essential for the intramolecular activation of its own kinase. PD-associated pathogenic RocCOR domain mutations (e.g. 1441C, Figure 2) increase GTP binding and decrease the rate of GTP hydrolysis. In contrast, the protective R1398H variant in the Roc domain has the opposite effect [17]. Once LRRK2 is GTP-bound, it is ready to undergo autophosphorylation and potentially phosphorylate other substrates to initiate neuronal cell signaling cascades. Once GTP is hydrolyzed to GDP, LRRK2 returns to its inactive state in which kinase activity is terminated (Figure 2) [18].

Several studies with related Roco proteins from prokaryotes and lower eukaryotes, especially from the bacteria *Chlorobium tepidum*, have contributed to our understanding of these processes [19–21].

LRRK2 can form dimers through its Roc-COR domain [22–24], but other studies suggest that the protein exists mainly as a monomer [25]. More recently, results from *in vivo* functional LRRK2 suggested that the protein cycles between a predominantly monomeric kinase-inactive form in the cytosol and a dimeric kinase-active form at the plasma membrane [26,27], while the Roco protein from *Chlorobium tepidum* is mainly dimeric in the nucleotide-free and GDP-bound states, and monomeric when bound to GTP [28] .

The LRRK2 kinase domain shows high similarity to stress-activated mixed lineage kinases [15,29], which are known to mediate cell-death in neurodegenerative diseases [30]. Moreover, the catalytic domain of LRRK2 kinase is similar to the catalytic domains of MAP kinase kinase kinases (MAPKKKs), suggesting that LRRK2 may be involved in MAP kinase signalling pathways [15,29,31–34]. LRRK2 was reported to phosphorylate different MAPKKs, MKK3/6 and MKK4/7 *in vitro*, and the LRRK2 G2019S kinase mutant is 3–4-fold more potent in phosphorylating MKK6 than WT LRRK2. Thus, MKKs represent potential targets, linking neuronal toxicity of PD-associated LRRK2 mutations to a molecular mechanism mediating cellular stress [35]. GTPases and MAPKKKs act normally upstream of multiple signal transduction cascades, suggesting that the multifunctional character of LRRK2 may play an important role in neuronal cell signaling. Therefore LRRK2 mutations may disturb this signaling, and subsequently enhance the pathogenesis of PD. The role of LRRK2 as a signaling protein was recently reviewed by us, and will not be further explored herein [36].

Other potential substrates of LRRK2 are members of the ERM family of proteins, including ezrin, moesin, and radixin [37].

Since most reports show that LRRK2 can phosphorylate its substrates only under *in vitro* conditions, it is remarkable that a subset of Ras-like GTPases, also named Rab-

GTPases, can be phosphorylated by LRRK2 both *in vitro* and *in vivo* [38,39], highlighting the role of LRRK2 on essential steps in vesicle trafficking processes.

Several studies addressed the physiological and pathological roles of LRRK2 and how they may relate to neurodegeneration, and found that LRRK1/LRRK2 double knock-out mice develop age-dependent neurodegeneration and lysosomal dysfunction [40]. An important connection between lysosomal dysfunction and sporadic PD is the high prevalence of PD in Gaucher's disease patients [41]. Thus, one can hypothesize that LRRK2 may cause lysosome-associated defects in PD.

Does LRRK2 interact with aSyn or tau?

Given the highly complex etiology of PD, and the fact that multiple genes have been associated with both familial and sporadic forms of the disease, the question of whether there is cross-talk between the various proteins encoded by the different genes is highly relevant.

Until now, it is still debatable if there is a direct interaction between aSyn and LRRK2. We have shown that WT and G2019S LRRK2 co-immunoprecipitate with aSyn in human cell lines, and that knocking-down LRRK2 resulted in a lower percentage of cells displaying aSyn inclusions. These findings suggest a possible interaction between both proteins, but do not definitively demonstrate a direct interaction [31,42]. LRRK2 was also shown to promote the formation of aSyn aggregates, and lead to age-dependent alterations in aSyn aggregation in kidneys, in murine *LRRK2* knockout models [43,44]. In contrast, co-expression of human LRRK2 and aSyn in transgenic mice did not alter aSyn pathology [45]. In mice overexpressing G2019S LRRK2, high LRRK2 levels improved motor skills independently of aSyn expression, pointing to a lack of interaction [45]. On the other hand, it has been reported that aged mice overexpressing this LRRK2 mutant showed higher levels of phosphoS129 aSyn, and

increased aSyn aggregation [46,47]. Interestingly, it has been found that in the A53T aSyn transgenic mouse, neurodegenerative phenotypes happen independently from LRRK2 expression, pointing again to a lack of interaction [48].

LRRK2 may affect aSyn aggregation through mechanisms that are still unclear, but one possibility would be by altering the activity of the endocytic pathway, affecting the internalization of aSyn.

LRRK2 might also modulate the levels and aggregation of aSyn in neurons by directly affecting the phosphorylation state of aSyn (section below), by phosphorylating proteins interacting with aSyn, or by affecting the subcellular distribution of aSyn ^[49].

LRRK2 and aSyn are both degraded by chaperone-mediated autophagy (CMA), as both proteins contain CMA recognition domains. Both proteins interact with LAMP2a and, a decrease in LAMP2a levels leads to the intracellular accumulation of aSyn and wild type (WT) LRRK2, but not on the G2019S LRRK2 mutant [50–53].

Another way in which both proteins may interact is via disruption of the equilibrium between mitochondrial fission and fusion and their effects on the respiratory chain. Both aSyn and LRRK2 may have an effect on the mitochondrial respiratory chain, specifically on the activity of the mitochondrial complex I [54,55]. This may lead to oxidative stress, a decrease in ATP production and/or a decrease in mitochondrial respiration [56–58]. Interestingly, in microglial cells, silencing of LRRK2 decreases autophagy and affects cell clearance [59,60].

It has been proposed that LRRK2 may exacerbate aSyn-induced microglial pathology and neuroinflammation, as LRRK2 may modulate the activation of microglia [61]. Because astrocytes and microglia participate in the clearance of aSyn, changes in responsiveness of these cells produced by LRRK2 may lead to alterations in the clearance of internalized aSyn [59]. This has not been as extensively studied in mice models. Nonetheless, one study shows that several months after adeno-associated

virus (AAV) injection, microglia is still not activated in WT and G2019S LRRK2 knock-in mice [61].

In summary, LRRK2 may affect aSyn aggregation through effects on endocytosis, autophagy and, consequently, lysosomal degradation of aSyn.

In the case of tau, it is also still controversial whether there is a direct interaction with LRRK2. Recent studies point to a possible interaction between the two proteins, as we and other have documented using co-immunoprecipitation experiments [62,63]. We have also observed that tau levels increase when co-expressed with LRRK2 in HEK293 cells [62]. Additionally, it has also been shown that G2019S knock-in mice and primary neuronal cultures showed increased tau phosphorylation [64,65]. Furthermore, mice overexpressing this LRRK2 mutant showed higher hippocampal neuron-to-neuron transmission of human tau [66]. On the other hand, double transgenic mouse models for mutant R1441G LRRK2 and P301S tau, showed no effect on tau hyperphosphorylation [67].

Toxic tau can be degraded via macroautophagy and CMA [68]. Tau contains two CMA recognition motifs, both in the microtubule binding domain [69]. Even though this would point to similar interactions to those seen with aSyn and LRRK2 in the CMA, it has also been shown that LRRK2 does not interfere with the autophagy-mediated degradation of tau, as treatment with autophagy inhibitors does not lead to changes in tau levels when it is co-expressed with LRRK2 [62]. Furthermore, it has been proposed that LRRK2 dysfunction influences how tau is processed post-translationally, affecting compartmentalization and the functional roles of tau in neuronal maintenance and transport [62]. In fact, it has been shown that LRRK2 promotes the accumulation of high molecular weight tau species pointing to a possible interaction between the two proteins [62]. Not only that, but LRRK2 is also related to pallido-ponto-nigral degeneration caused by the tau N279K mutation, where a strong expression of

inflammatory molecules in astrocytes and microglia was seen [70]. In a similar way to aSyn, there is few literature showing direct interaction between LRRK2 and tau.

aSyn and tau are phosphoproteins

Phosphorylation is a common posttranslational modification (PTM) that regulates, like all PTMs, protein structure, localization, and function.

aSyn can be phosphorylated in different residues, such as Ser129, Ser87, or Tyr125, but the precise biological effects of aSyn phosphorylation are still unclear [71,72]. In LBs, aSyn is highly phosphorylated on Ser129 and Ser87, but whether phosphorylation enhances or protects against aggregation remains unclear and controversial [73–75]. Likewise, tau is also known to be heavily phosphorylated in neurofibrillary tangles in AD, progressive supranuclear palsy, Pick's disease and FTD [75–78]. Tau has more than 80 phosphorylation sites (serine, threonine, and tyrosine residues) in its longest isoform [79]. Most of these phosphorylation sites are found in the microtubule-binding domain of the protein, near the C-terminus [79]. According to the numbering of the longest human brain tau isoform, prominent phosphorylation sites are serines 202, 214, 235, 262, 356, 396, 404 and 422, and threonines 181, 205, 212 and 231 [80]. Interestingly, phosphorylation at Thr205 and Ser262 are characteristics of AD and phosphorylation at Thr181 is a marker of several tau-associated dementias, while phosphorylations at residues Ser396 and Ser404 are considered markers of paired helical filaments and participate in the binding to microtubules [81–86].

Thus, identifying the kinases that phosphorylate aSyn and tau is of great interest, as kinase inhibition is regarded as a possible therapeutic strategy [87]. Indeed, several kinases capable of phosphorylating aSyn and tau have already been identified, like PLK-1, PLK-2, PLK-3 and GSK3 [88,89], but we still do not know if these are the most relevant kinases from a physiological perspective. Given that LRRK2 has kinase

activity, the question of whether aSyn or tau can be phosphorylated by LRRK2 attracted immediate attention.

Does LRRK2 phosphorylate aSyn or tau?

There are more than 40 variations in LRRK2, eight of which (N1437H, R1441C/G/H/S, Y1699C, G2019S, and I2020T) segregate with PD [90,91], and four (A419V, R1628P, M1646T and G2385R) that are considered to reduce the risk for PD [92–95]. The G2019S is among the most studied LRRK2 mutations, and it is known to increase the intrinsic kinase activity of LRRK2 [36]. Most of the pathogenic LRRK2 mutants are phosphorylated at S1292 due to an autophosphorylation process [36].

LRRK2 coprecipitates with aSyn in diffuse Lewy body disease samples and has been reported to directly phosphorylate aSyn at S129 at low levels *in vitro*. However, phosphorylation levels are increased several fold by the G2019S mutation [96]. In the mouse striatum, LRRK2 and aSyn are co-regulated, even though a direct interaction was not been found in the same study [97]. Other authors failed to detect the phosphorylation of aSyn on S129 by LRRK2 [43,45].

Further *in vitro* and *in vivo* experiments also failed to show direct phosphorylation of aSyn by LRRK2 [98], or even found reduced phosphorylation of aSyn upon co-expression of LRRK2 [43]. Nevertheless, one study reported that supernatants of cells overexpressing different LRRK2 kinase domains induced the phosphorylation of recombinant aSyn on S129 [96]. This study, however, has not been replicated by other groups.

In the case of tau, it has been proposed that WT LRRK2 can phosphorylate monomeric tau *in vitro* [63]. Additional *in vitro* studies showed that PD-associated mutations in LRRK2 induce a progressive reduction in neuronal neurite length and the formation of phospho-tau inclusions, suggesting that tau phosphorylation may be directly induced

by LRRK2. However, this could not be proven [99,100]. Interestingly, the overexpression of G2019S LRRK2 in *Drosophila* induces tau phosphorylation at position T212 and changes tau localization to dendrites by recruiting GSK3 β [101]. Additionally, in SH-SY5Y cells stably expressing LRRK2, phosphorylation of tau by LRRK2 mainly happens when both proteins are bound to microtubules, normally leading to the phosphorylation of the Thr181, but not when the proteins are free in the cytoplasm [102,103]. This may reflect the translocation of LRRK2 on skein-like cytoplasmic pools under certain conditions, an observation which has not been fully characterized [104].

In primary dopaminergic neurons, the G2019S mutant led to hyperphosphorylation of residues Thr212 and Ser214 [101]. The phosphorylation of microtubule-bound LRRK2 happens by the recruitment of autoactivated GSK3 β , and GSK3 β mediates the activity of G2019S in dendrite degeneration [101]. However, it was hypothesized that LRRK2 may not interact with non-tubulin-associated tau [103].

Another mutation that increases LRRK2-mediated tau phosphorylation is R1628P. This mutant of LRRK2 is phosphorylated by Cdk5, leading to cell death [63,105]. In fact, GSK3 β and Cdk5 mediate the increase in tau phosphorylation by LRRK2. It has been suggested that Cdk5 phosphorylates tau at Ser396 and Ser404 after recruitment by LRRK2 in SH-SY5Y cells and, in mouse brain, GSK3 β coimmunoprecipitates with endogenous LRRK2 [63,106]. Furthermore, Cdk5 can also phosphorylate LRRK2 on Ser1627 leading to an increase in LRRK2 activity [105].

In summary, it is still not clear whether WT LRRK2 can directly phosphorylate aSyn, as only one study showed direct phosphorylation. In its mutant forms, it seems that LRRK2 may, at least in certain conditions, phosphorylate monomeric tau and aSyn. However, it is possible that phosphorylation may be indirect, through the activation of other kinases.

Rab GTPases – a common link between LRRK2 and aSyn and tau

As described above, LRRK2, aSyn and tau, are associated with familial and sporadic forms of PD, suggesting the interplay between these proteins (Figure 4) may contribute to disease progression. One possible scenario would be that LRRK2 could act as an upstream modulator, regulating the toxic effects of aSyn and tau via its kinase activity [107]. Since the direct phosphorylation of aSyn or tau by LRRK2 is still controversial, it is most likely that the observed effects are mediated by an intermediate substrate of LRRK2 that acts upstream of tau and aSyn.

The most promising class of LRRK2 intermediate substrates are Rab-GTPases (Rabs), also because some Rab proteins are candidate risk factors for sporadic PD and the interactions with specific Rab family members are functionally important in the context of LRRK2 function [108].

Rabs can be phosphorylated by LRRK2 in a conserved residue in their switch-II domains, which inhibits their interaction with GDP-dissociation factors (GDIs) and guanine nucleotide exchange factors (GEFs), and maintains them in their GDP-bound form on membranes [38,109]. Rab3A,B,C, Rab8A,B, Rab10, Rab12, Rab35 and Rab43 are currently considered the main endogenous LRRK2 phosphorylation substrates [110]. The Rab-family is part of the Ras superfamily of small GTPases, monomeric G-proteins that have been evolutionally conserved in eukaryotes, and contains more than 60 members in humans. Rabs are key regulators of membrane trafficking, vesicle formation and membrane fusion processes [111,112].

LRRK2 was found to exert negative effects on Rab7, inducing a delay in trafficking of the epidermal growth factor receptor (EGFR) [113]. Rab7 is involved in autophagy, as well in the endo-lysosomal pathway [114], and plays a central role in the maturation of the autophagosome [115].

Interestingly, Rab7 was found to increase the amount of aSyn in acidic compartments and to reduce its aggregation in a cell model [116].

Secretion of tau is also regulated by Rab7, as shown by the knock down of Rab7, which reduces the levels of secretion of hypo-phosphorylated tau [117]. On the other hand, LRRK2 increases tau secretion, without changing in the phosphorylation state of tau [62]. These findings provide important clues on the effects of the interplay between LRRK2 and one of its authentic downstream substrates, Rab7, and how this may impact on the secretion and/or degradation of tau and aSyn.

Interestingly, one study reported that the LRRK2 G2019S mutation enhances the propagation of aSyn via Rab35 phosphorylation in *C.elegans* [118]. Interestingly, the Rab35/ESCRT pathway is also essential for tau degradation [119].

In addition to the effect of Rab7 on aSyn, other associations between Rab GTPases and aSyn have been reported. For example, abnormal interactions with Rab3A, Rab5A and Rab8A were reported in transgenic mice overexpressing A30P aSyn [120–122]. We have also reported interactions between aSyn and other Rab proteins [123–126]. LRRK2 can also be controlled by the action of Rab29 (Rab7L1) [127–129], where exogenously expressed Rab29 recruits LRRK2 to the trans-Golgi network, and activates LRRK2, consequently enhancing the phosphorylation of Rab10 and Rab 8 [110,130]. Furthermore, LRRK2 can phosphorylate Rab29 on Ser71 and Thr72, abolishing the activity of Rab29, probably representing a negative feedback regulation of LRRK2 [128].

Another study reported a strong interplay between Rab29, LRRK2 and Rab8/10 [131]. When lysosomes were exposed to chloroquine, Rab29 moved to swollen lysosomes, recruiting LRRK2, which led to the phosphorylation of Rab8 and Rab10. Deletion of all three proteins showed that Rab8 activity kept the lysosomes small, whereas Rab10 promoted lysosomal exocytosis. Therefore, LRRK2 is thought to alleviate lysosomal

overload by stimulating the release of lysosomal content from the cells, which is controlled by Rab29 activity.

Certain Rab proteins, such as Rab1A, Rab7, Rab8A, Rab10, Rab11A, were found to be colocalized with aSyn aggregates and, upon overexpression, they can reduce aSyn aggregation in cellular systems. Potentially, these effects may be susceptible for negative modulation by LRRK2 phosphorylation [38,116,125,126,132–134].

Conclusion

Accumulating evidence supports an interplay between LRRK2 and aSyn, and between LRRK2 and tau. In turn, aSyn and tau may also interact in pathological conditions, suggesting that the three proteins may be “partners in crime”. The puzzle is complex, and we may still lack the complete set of pieces, but the emerging patterns suggest that LRRK2 may, directly or indirectly, affect the accumulation of both proteins. Despite the fact that aSyn and tau are phosphoproteins, and that LRRK2 has kinase activity, current evidence suggests that LRRK2 may not phosphorylate the two proteins directly. It appears more likely that the effect of LRRK2 on aSyn and tau may take place via the modulation of other key proteins, such as RabGTPases or other kinases, which would in turn modulate the biology of the two proteins. LRRK2 may, therefore, affect pathways involved in the degradation of aSyn and tau, affecting also the propagation of pathology, resulting in PD-associated features. The reasons for this are not fully understood at the present moment, and need to be further investigated, as this knowledge may culminate with the identification of novel strategies for therapeutic intervention.

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Abbreviations:

ANK: ankyrin repeats
ARM: armadillo repeats
COR: C-terminal of roc
LRR: leucine rich repeats
MT: microtubules
PD: Parkinson's disease
Roc: ras of complex proteins

Conflicts of interest

The authors declare no conflict of interest

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Figure legends

Figure 1. Schematic representation of aSyn. aSyn can be divided in three domains. PD-associated mutations seem to cluster in the N-terminal region of aSyn, which is important for membrane interactions. S129 is one of the phosphosites identified in aSyn, and its localization is in the C-terminal region of the protein.

Figure 2. Schematic representation of LRRK2. LRRK2 is a large and complex protein, with various domains. The GTPase activity of LRRK2 leads to its activation, which increases its kinase activity and ability to phosphorylate several RabGTPases. The most common PD-associated LRRK2 mutation, G2019S, increases the kinase activity of LRRK2, thereby affecting several important pathways such as autophagy and vesicular trafficking, and may lead to lysosomal dysfunction.

Figure 3. Schematic representation of tau. Tau can exist in multiple isoforms, depending on the presence of different repeat regions (R1 to R4). Tau has numerous phosphorylation sites, rendering the protein a substrate for various kinases. Tau interacts with several cellular components, and hyperphosphorylation may modulate these interactions, possibly disrupting microtubules. Different tau mutants have been associated with familial forms of neurodegenerative disorders in which tau accumulates.

Figure 4. The possible triangle between LRRK2, aSyn and tau. We still do not fully understand how LRRK2 may affect the biology/pathobiology of aSyn and/or tau. One possibility is that, by phosphorylating Rab proteins, LRRK2 may affect the biology of aSyn, especially in what concerns membrane interactions. In the case of tau, LRRK2 may affect the activity of signaling pathways that affect the phosphorylation of tau, thereby affecting its interaction with microtubules. Ultimately, LRRK2 may affect aSyn and tau levels by affecting the degradation of the two proteins, which could, in turn, affect also the spreading of aSyn and/or tau pathology. Future studies will be important to clarify whether the three proteins are partners in crime or simply unfortunate bystanders during pathological processes that lead, for example, to PD.

Mutations/Risk factors

**Membrane phospholipid
binding site**

**Promotes
aggregation**

Chaperone function

Functions

Pro-aggregation mutant

VQIINK~~280~~

VQIVYK₃₁₁

Anti-aggregation mutant

VQPINK~~280~~

VQPVYK₃₁₁

Projection domain

Microtubule assembly domain

C-tail

p150/Dynactin
Annexin A2
Plasma membrane

Fyn, Src, PI3K
SH3-domains,
PIN1

Microtubules, PP2A,
Calmodulin, Aggregation

Interactions

LRRK2

Rabs

Membranes

Microtubule

?

aSyn

Tau

**Synaptic
vesicle release**

**Destabilized
microtubules**

Degradation

?