

AWARD NUMBER: W81XWH-19-1-0264

TITLE: Role of Lipid Dyshomeostasis in Cognitive Dysfunction of Parkinson's Disease

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REPORT DATE: AUGUST 2020

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE		Form Approved OMB No. 0704-0188
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1. REPORT DATE: AUGUST 2020	2. REPORT TYPE: Annual	3. DATES COVERED: 15JUL2019 - 14JUL2020
4. TITLE AND SUBTITLE: Role of Lipid Dyshomeostasis in Cognitive Dysfunction of Parkinson's Disease		5a. CONTRACT NUMBER: W81XWH-19-1-0264
		5b. GRANT NUMBER: PD180058
		5c. PROGRAM ELEMENT NUMBER:
6. AUTHOR(S): Vidyadhara D J E-Mail:vidyadhara.dj@yale.edu		5d. PROJECT NUMBER:
		5e. TASK NUMBER:
		5f. WORK UNIT NUMBER:
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Yale University Rebecca Balentin 105 Wall St New Haven Ct 06511-8917		8. PERFORMING ORGANIZATION REPORT NUMBER: 4B992
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012		10. SPONSOR/MONITOR'S ACRONYM(S) USAMRAA
		11. SPONSOR/MONITOR'S NUMBER(S)
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited		
13. SUPPLEMENTARY NOTES		

14. ABSTRACT:

Lipid dyshomeostasis plays a prominent role in many cognitive disorders envisaging its contribution to cognitive dysfunction of Parkinson's disease (PD). Mutations in *GBA* that encodes glucocerebrosidase 1 (Gcase1) results in accumulation of glycosphingolipids (GSLs) leading to Gaucher disease (GD) and also the most common genetic risk factor for PD. Patients with homozygous *GBA* mutations exhibit PD accompanied by severe cognitive deterioration compared to sporadic cases. Even the heterozygous *GBA* carriers (who do not have GD) are at intermediate risk for developing cognitive dysfunction. A recent study in our lab revealed that the accumulation of glucosylceramide (GlcCer) and glucosylsphingosine (GlcSph) promotes alpha-synuclein aggregation in vitro. Additionally, a mouse line obtained by crossbreeding novel long-lived mouse model of GD (*Gba* L444P KO) with alpha-synuclein transgenic PD mice (*SNCA* tg) featured accelerated PD progression including motor abnormalities (*Gba*/*SNCA* or GD/PD mice) alongside accumulated GSLs. In this study, we are evaluating if the similar accumulation of GSLs and subsequent cellular and circuit level disturbances in the hippocampus and cortical areas drive cognitive dysfunction in *GBA*-associated PD. To achieve this, We are using our well-established mouse models of GD (*Gba* L444P KO), PD (*SNCA* tg) and GD/PD (*Gba*/*SNCA*) mice along with wildtype controls. Considerable amount of time was spent on establishing these mice colonies in first year. We performed both motor and cognitive behavior assays longitudinally at 3, 6 and 9 months of age on these mice. Motor behavior analysis indicate GD/PD mice to have a severe progressive motor deficit compared GD mice. Results of cognitive behavior assay are being analysed. We standardized immunohistochemistry, confocal microscopy and image analysis methods as well as collected and stored the mice brain specimens at appropriate age for upcoming experiments. Along with publishing a review article, PI was also involved in various professional development and training activities. During the next reporting period, we will perform lipidomics and histopathological studies to correlate with our behavior experiments and identify time course of pathological events with respect to the onset of cognitive dysfunction. We will also perform functional analysis of hippocampus through electrophysiology and conduct sufficiency experiments to evaluate our proposed mechanism of GlcSph and GlcCer mediating cognitive dysfunction in PD. Thus, our study will provide novel insights into the role of GSLs in deciphering cortico-hippocampal dysfunction and associated cognitive decline in PD.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:**a. REPORT**

Unclassified

b. ABSTRACT

Unclassified

c. THIS PAGE

Unclassified

**17. LIMITATION
OF ABSTRACT**

Unclassified

**18.
NUMBER
OF PAGES**

36

**19a. NAME OF
RESPONSIBLE PERSON
USAMRMC****19b. TELEPHONE NUMBER**
(include area code)Standard Form 298
(Rev. 8-98)

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1. INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

More than 80% of people with Parkinson's disease (PD) suffer from cognitive dysfunction, which is considered as one of the major unmet needs by the PD patients and their caregivers. Still, little is known regarding the causative mechanisms from circuit to cellular level. Lipid dyshomeostasis is implicated in a growing number of cognitive disorders, envisaging its contribution to cognitive dysfunction associated with PD. Mutations in *GBA* gene that encodes glucocerebrosidase 1 leading to accumulation of glycosphingolipids (GSLs), is the most common genetic risk factor for PD. Cognitive dysfunction is more prevalent and severe in PD patients with *GBA* mutations. Here, we propose to use our well-established mice models of *Gba* mutations with PD phenotype to decipher the role of lipid dyshomeostasis and its mechanisms leading to cognitive dysfunction of PD.

2. KEYWORDS: Provide a brief list of keywords (limit to 20 words).

Parkinson's disease, cognitive dysfunctions, *GBA*, Lipid dyshomeostasis, glycosphingolipids, glucocerebrosidase 1

3. ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1: To characterize the cortical and hippocampal lipid profile in the brains of *Gba* KO, *SNCA* tg and *Gba*/*SNCA* mice as a function of age and correlate it with α -synuclein pathology. (n=5/mice line/time point/experiment)

Major Task 1 : Lipidomics for glycosphingolipids and phospholipids in *Gba* L444P KO, *SNCA* tg and *Gba*/*SNCA* mice at 3, 6, 9 and 12 months of age (1-18 months of project period)

Major Task 2: Immunohistochemistry in *Gba* L444P KO, *SNCA* tg and *Gba*/*SNCA* mice of 3, 6, 9 and 12 months of age (1-24 months)

Major Task 3: Western blotting (1-14 months)

Milestones achieved: Mice required for the above experiments are being bred and aged. Genome scanning to confirm the background mice strain for *Gba* L444 KO was performed. 25% of lipidomics is being performed. 25% of mice brain specimens required for immunohistochemistry are collected.

Specific Aim 2: To assess the cognitive capabilities of *Gba* KO and *SNCA* tg mice and to evaluate if it worsens in *Gba*/*SNCA* mice (n=12/mice line/time point/experiment) (3-15 months)

Milestones achieved: WT (wild type) and *SNCA* tg mice (PD mice) are bred and aged for the above experiment. We continued longitudinal behavioral assay (both motor and cognitive behavior) for *Gba* L444P KO (n=12) and *Gba*/*SNCA* mice (n=13) (GD and GD/PD mice, respectively) at 3, 6 & 9 months of age.

Specific Aim 3: *Evaluation of LTP in Schaffer collateral synapses of hippocampal sections and to investigate if AAV-shRNA mediated inhibition of Asah1 and Ugcg ameliorate hippocampal pathology and cognitive deficits in Gba KO, SNCA tg and Gba/SNCA mice (16-24 months)*

Milestones achieved: Procured required instrumentation for stereotaxic hippocampal injections to perform AAV-shRNA mediated inhibition of Asah1 and Ugcg. We have started the process of preparing/procuring shRNA for mouse ASAH1 and UGCG recombined with AAV9 for hippocampal injections.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

As our complete proposal rely on animal experiments, major part of first quarter was spent on obtaining Animal Care and Use Review Office (ACURO) approval and setting up the mice breeding colonies. We also published a review article in the first quarter related to the topic of interest of this grant titled “Role of the endolysosomal system in Parkinson's disease” (attached). This was received well among the research community and one of the most read article in the Journal of Neurochemistry for the year 2019-20 (10 citations).

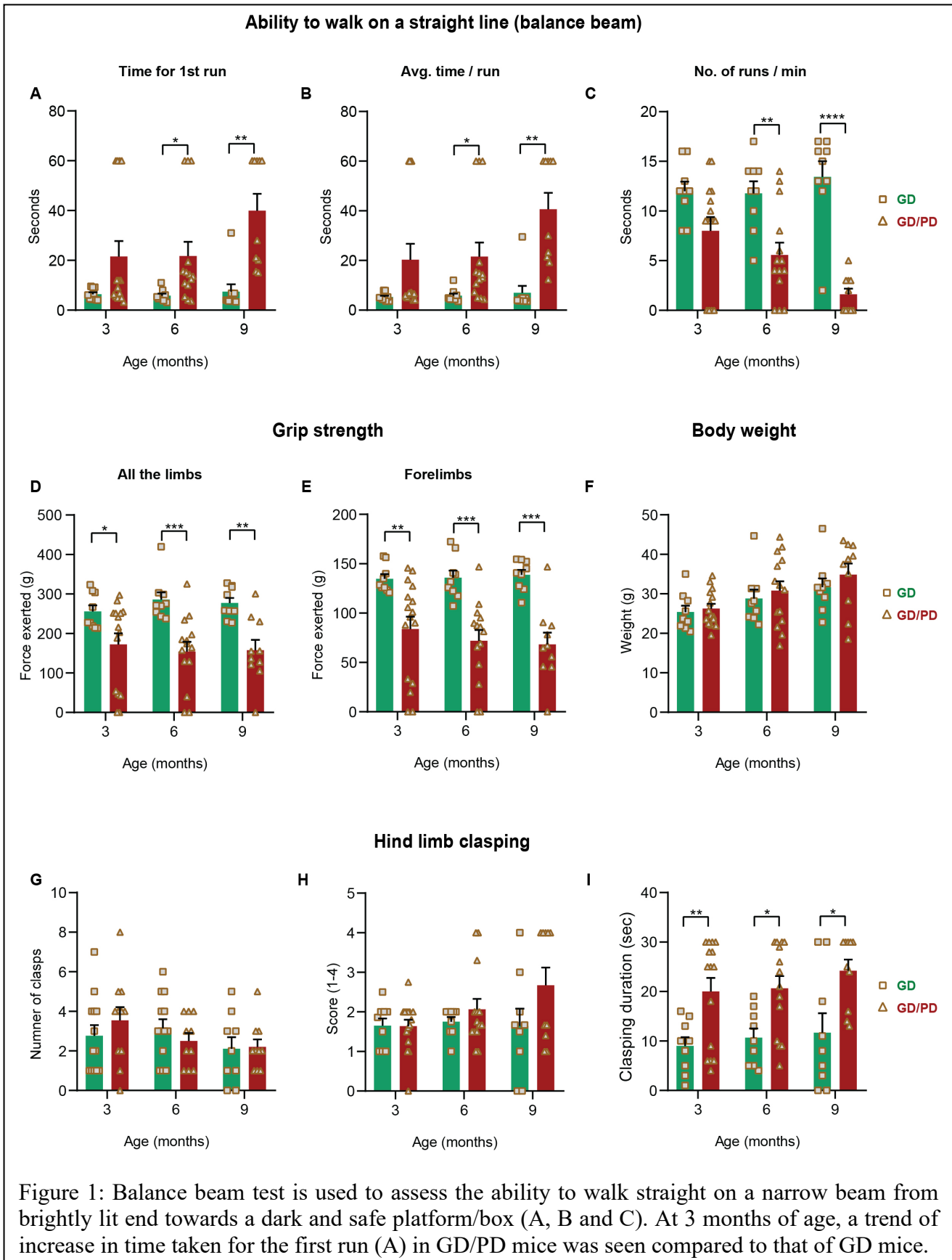
In the second quarter, we obtained *Gba* L444P KO (GD) mice and *Gba*/SNCA (GD/PD) mice breeding pairs (total 8) from Mistry Lab, Yale University School of Medicine, New Haven, USA. We confirmed the background strain of GD mice to be C57BL/6J by genome scanning, which would be used as WT control. We standardized the genotyping protocol for all the mice lines. About 70% of GD and GD/PD mice have been raised and aged in our breeding colony and are being used for the experiments. SNCA tg (PD) and WT mice are being bred to obtain required number of mice for this project.

Specific Aim 1: *To characterize the cortical and hippocampal lipid profile in the brains of Gba KO, SNCA tg and Gba/SNCA mice as a function of age and correlate it with α -synuclein pathology. (n=5/mice line/time point/experiment)*

Lipidomics is being performed for the 12 months cohort of *Gba* KO, SNCA tg, *Gba*/SNCA and WT mice. We standardized certain techniques like immunohistochemistry, confocal microscopy and image analysis.

Specific Aim 2: *To assess the cognitive capabilities of Gba KO (GD) and SNCA tg mice (PD) and to evaluate if it worsens in Gba/SNCA mice (GD/PD) (n=12/mice line/time point) (3-15 months)*

Behavior evaluation: As *Gba* KO (GD, n=9) and *Gba*/SNCA mice (GD/PD, n=15) were available quicker, we performed both motor and cognitive behavior evaluation on these mice. Results so far suggest GD/PD mice to have exacerbated motor dysfunctions compared to that of GD mice which also worsens with age. Novel Object recognition test to assess cognitive behavior was also performed at 3, 6 & 9 months of age, where data is being analyzed currently. Once we perform behavior evaluation on age-matched WT control and SNCA tg (PD) mice, we will have better picture on these observations. Below are the results for motor behavior analysis.



A significant difference was noted at 6 months (A, $*p<0.05$) which was further worsened at 9 months of age (A, $**p<0.01$). Similar progressive increase in average time taken per run (B) was noted in GD/PD mice compared to that of GD mice (B, 6 months, $*p<0.05$; 9 months, $**p<0.01$). Progress in motor deficits was more apparent when the number of runs these mice can perform in a minute (C) was quantified. GD/PD mice progressively lost their ability to run from 3 to 9 months of age compared to that of GD mice (C, 6 months, $**p<0.01$; 9 months, $****p<0.001$). Thus, our balance beam assay suggests GD/PD mice to develop severe motor deficits with age, whereas GD mice maintained their motor abilities across age groups.

Grip strength is a measure of force exerted by the animal in grasping specially designed pull bar assembly of a grip strength meter. A significant loss of strength was noted as early as 3 months and sustained with aging in GD/PD mice compared to GD mice, both in all the limbs (D, 3 months, $*p<0.05$; 6 months, $***p<0.001$; 9 months, $**p<0.01$) and the forelimbs (E, 3 months, $**p<0.01$; 6 months, $***p<0.001$; 9 months, $***p<0.001$). No significant difference was seen in body weight (F).

Mice, when picked up by the tail and made to descend towards the ground, extend their limbs reflexly in anticipation of contact. Mice with certain neurological dysfunction display hind limb claspings instead of extension. We made mice to perform this maneuver for 30 seconds and scored the hindlimb clasps (0: no clasp; 1: One hind limb clasp; 2: both the hind limbs clasp; 3: Hind limbs and trunk clasp; 4: Hind limbs and forelimbs clasp). Both GD and GD/PD mice revealed severe hind limb claspings, which we will be able to interpret better when we add WT mice. When we compared GD and GD/PD mice, no significant difference was noted across age groups in number of clasps (G) and the clasping score (H), though clasping appeared to be more severe in GD/PD mice at 9 months of age (H, higher number of score 4 clasps). Interestingly, clasping duration was higher in GD/PD mice across all the age groups compared to GD mice, suggestive of profound motor deficits (I, 3 months, $**p<0.01$; 6 months, $*p<0.05$; 9 months, $*p<0.05$).

Our motor behavior analysis thus indicate GD/PD mice (which has both *GBA* L444P KO and A30P *SNCA* mutations) to have severe motor deficits than GD mice (with only *GBA* L444P KO mutation).

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Training: PI was trained in novel object recognition behavior assay to assess learning and memory, laser scanning confocal microscopy, electron microscopy, synaptosome preparation and CRISPR. PI established successful collaboration with Dr. Mistry lab at Yale who are helping him with the complicated genotyping methods related to this project. PI also regularly interacts with Dr. Pramod Mistry who is a leader in *GBA*-related studies and his lab members through joint lab meetings. This is helping him develop a clinical perspective on *GBA*-associated PD. Dr. Sreeganga Chandra, who

is the mentor in this project interacts with PI one-on-one every week and helps him with the resources and provide suggestions on the techniques and concepts. Overall administrative process to get all the regulatory approvals and utilizing the grant money in compliance with the terms was also a learning experience for the PI.

Professional development: PI had a detailed discussion with his mentor Dr. Chandra on his career interests and professional development plan in the beginning of calendar year 2020. PI also mentored and trained two undergraduate research assistants. PI is selected to be the guest editor for 'Neurodegenerative disorders' section in the Journal of Visualized Experiments (JoVE). He has completed 80% Certificate of College Teaching Preparation (CCTP) offered at Yale Center for Teaching and Learning. PI has attended career development workshops conducted by Yale Postdoctoral Association, Kavli Neuroscience networking activity, and the departmental workshops on technical and conceptual skills pertaining to neuroscience and PD research. PI is also an active committee member of SYNAPSES seminar series, Dept. of Neuroscience postdoc committee and for the committee on Diversity, Equity, and Inclusion (DEI). He has actively participated in Yale Parkinson's Disease Research Group, a research seminar series on PD from clinical and basic research perspectives. Attended summer course on Biomedical Data Science & Health Informatics, 2020. Attended all the virtual 3P: Parkinson Postdoc Program seminars organized by The Cure Parkinson's Trust. PI has also attended virtually the Monsoon Brain Meeting in the month of June and YPA's 3rd Annual Postdoc Symposium in July 2020. PI has registered to attend the virtual International Congress of Parkinson's Disease and Movement Disorders, 2020 in the month of September.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

PI and the mentor published an open access review article related to the project topic in the "Journal of Neurochemistry", which is one of the most downloaded articles in that journal for 2019-2020 (attached). PI and the mentor also wrote a lay article on this topic as a part of World Brain Day 2020, to be published in an outreach program from the dept. of Neurology at Yale. PI discussed the developments of the project with Dr. Pramod Mistry and his lab members, who are working extensively on Gaucher Disease and other *GBA*-related disorders.

What do you plan to do during the next reporting period to accomplish the goals?

If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

1. Lipidomics for GSLs and phospholipids in *Gba* L444P KO (GD), *SNCA* tg (PD), *Gba/SNCA* (GD/PD) and WT mice at early and late symptomatic stage will be performed. We will validate the hits we get in lipidomics by immunohistochemistry and Western blotting. We will use these techniques to also assess how perturbances in lipid levels influence the α -synuclein pathology and neuronal, glial, and synaptic health in the cognitive brain areas. This will cover the objectives of specific aim 1.

2. As a part of specific aim 2, we will analyze the accumulated data of Novel Object Recognition for GD and GD/PD mice. We will continue to assess these mice at 12 months of age, along with longitudinal cognitive behavioral assessment of PD (*SNCA* tg) and WT mice from 3 to 12 months of age. We will continue to monitor motor behavior in parallel to cognitive behavior. Correlating this with lipidomics and histopathological studies will allow us to identify the time course of pathological events and the onset of cognitive dysfunction.

3. We will evaluate these mice at 6 months of age for hippocampal Long Term Potentiation (LTP). This is widely used to assess hippocampal functions that can be directly correlated to biological and behavioral phenotypes. We will perform this experiment in collaboration with Dr. Pablo E. Castillo, Albert Einstein College of Medicine, New York. Also, sufficiency experiments for the proposed mechanism of GlcSph and GlcCer mediating cognitive dysfunction in PD will be assessed by Adeno-Associated Virus (AAV)-shRNA-mediated silencing of *Asah1* and *Ugcg*, genes that form enzymes responsible for the biosynthesis of these two GSLs. We will assess whether this silencing successfully restores hippocampal health and cognitive function. These experiments will complete the goals of our specific aim 3.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

PD is diagnosed through motor abnormalities, and within several years, 80% of patients develop cognitive problems. Knowing this statistic provides a window for preventive measures but understanding the mechanisms through which cognitive problems arise is crucial. This work will provide major breakthroughs in this area and identify lipids as important modifiers of cognitive dysfunction in PD. Future therapies may be developed targeting lipid dyshomeostasis to prevent cognitive dysfunction in PD patients, which is considered one of the main unmet needs. There are already several drugs commercially available that blocks accumulation of glycosphingolipids (GSLs) such as GlcSph in cancer. Drugs targeting GlcCer are under clinical trials for GD. These two GSLs are the key targets of our study. If we are able to successfully establish the role of these in cognitive dysfunction of PD, those drugs can be repurposed to be used in PD, which would help patients in the near future. This study will also provide evidence to support if *Ugcg* and *Asah1* which produce GSLs can be the potential targets to treat cognitive decline in PD.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

GBA mutations are also known to increase risk for Dementia with Lewy Bodies (DLB), which is closely related to PD. Thus, our work will broaden the therapeutic landscape for treatment of cognitive dysfunction also in other synucleinopathies such as DLB, along with unravelling the causative mechanisms.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- transfer of results to entities in government or industry;
- instances where the research has led to the initiation of a start-up company; or
- adoption of new practices.

Through our experiments, we have helped standardizing open field and novel object recognition assays for mice in the recently opened Kavli Rodent Behavior Analysis facility at the dept. of Neuroscience, Yale School of Medicine.

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- improving public knowledge, attitudes, skills, and abilities;
- changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or
- improving social, economic, civic, or environmental conditions.

Even after more than 200 years of its first description, PD remains predominantly a movement disorder even in the public domain. It is important to sensitize the caregivers and the community about the immense cognitive and psychiatric disturbances the patients go through. We will communicate the intent and the results of our study to the public in lay words in all the available digital and print platforms.

- 5. CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

In first quarter, we obtained congruency certificate from Yale University's Institutional Animal Care and Use Committee (IACUC) that this proposal is in congruent with the IACUC protocol number 2018-11117. However, Animal Program Oversight Specialist at ACURO identified that the two behavioral experiments we have proposed in this grant were not approved in the IACUC protocol. Based on these suggestions, we modified our IACUC protocol and obtained ACURO approval. This delayed starting the experiments. Research activities were completely shutdown from 16th March to June 31st, 2020 due to Covid-19 pandemic. This severely affected our planned experiments in aim 2, such as immunohistochemistry and lipidomics, and also standardizing experiments in aim 3. Setting up new breeding colonies to obtain certain mice that are critical for this grant was also affected. Our lab is still in Phase 2 reactivation with limited access. Extra-time available off the bench was used to complete 80% of "Certificate of College Teaching Preparation" which is a part of researcher development plan. PI also participated in virtual career and professional development activities during this period. Based on the progress we could make in the next reporting period, we might ask for a No-Cost Extension.

We found that *Gba/SNCA* (GD/PD) mice develop motor deficits at an early time point in our experiment (3 months of age). Thus, we did not include Morris water maze (MWM) cognitive behavior assay as motor abnormalities affecting swimming abilities would lead to confounding results in cognitive evaluation by MWM. We have added extra parameters to novel object recognition test so that we do not compromise on our ability to assess the cognitive behavior.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Complete shutdown of our laboratory due to Covid-19 pandemic from 16th March to June 31st, 2020 along with limited access to the facilities during the following months have significantly delayed our experiments. Because of this, we could not use 35-40% of the allotted grant for the budget period 1. This also includes grant that was allotted for travel and attending meetings. We plan to utilize the remaining budget in the next reporting period.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and

reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

D. J. Vidyadhara, John E. Lee & Sreeganga S. Chandra; Role of the endolysosomal system in Parkinson's disease. *Journal of Neurochemistry*, 2019, Volume 150, Issue 5, Pages 487-506, <https://doi.org/10.1111/jnc.14820> (published); acknowledgement of federal support (Yes)

Books or other non-periodical, one-time publications. Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothing to report.

Other publications, conference papers and presentations. Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

Nothing to report.

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- data or databases;
- physical collections;
- audio or video products;
- software;
- models;
- educational aids or curricula;
- instruments or equipment;
- research material (e.g., Germplasm; cell lines, DNA probes, animal models);
- clinical interventions;
- new business creation; and
- other.

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Name: Vidyadhara D J, PhD
Project Role: PI
Researcher Identifier (e.g. ORCID ID): <https://orcid.org/0000-0003-0974-0307>
Nearest person month worked: 12
Contribution to Project: Dr. Vidyadhara has obtained Yale IACUC and ACURO approvals, procured mice and set-up breeding colonies, all the experiments, data analysis, purchase of consumables and prepared necessary progress reports.

Name: Sreeganga S. Chandra, PhD
Project Role: Mentor
Researcher Identifier (e.g. ORCID ID): <https://orcid.org/0000-0001-9035-1733>
Nearest person month worked: 12
Contribution to Project: Dr. Chandra has obtained IACUC approval for her proposal, number 2018-11117 which is in congruent with this proposal. Dr. Chandra has also mentored PI at all the stages of experiments towards successful execution of the grant.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report.

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support,

supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner's contribution to the project (identify one or more)

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);
- Facilities (e.g., project staff use the partner's facilities for project activities);
- Collaboration (e.g., partner's staff work with project staff on the project);
- Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and
- Other.

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.

Not applicable

QUAD CHARTS: If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.

Not Applicable

- 9. APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Attached journal article: D. J. Vidyadhara, John E. Lee & Sreeganga S. Chandra; Role of the endolysosomal system in Parkinson's disease. *Journal of Neurochemistry*, 2019, Volume 150, Issue 5, Pages 487-506, <https://doi.org/10.1111/jnc.14820>

REVIEW

Role of the endolysosomal system in Parkinson's disease

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Abstract

Parkinson's disease (PD) is one of the most common neurodegenerative disorders, affecting 1–1.5% of the total population. While progress has been made in understanding the neurodegenerative mechanisms that lead to cell death in late stages of PD, mechanisms for early, causal pathogenic events are still elusive. Recent developments in PD genetics increasingly point at endolysosomal (E-L) system dysfunction as the early pathomechanism and key pathway affected in PD. Clathrin-mediated synaptic endocytosis, an integral part of the neuronal E-L system, is probably the main early target as evident in auxilin, RME-8, and synaptotagmin-1 mutations that cause PD. Autophagy, another important pathway in the E-L system, is crucial in maintaining proteostasis and a healthy mitochondrial pool, especially in neurons considering their inability to divide and requirement to function an entire life-time. *PINK1* and *Parkin* mutations severely

perturb autophagy of dysfunctional mitochondria (mitophagy), both in the cell body and synaptic terminals of dopaminergic neurons, leading to PD. Endolysosomal sorting and trafficking is also crucial, which is complex in multi-compartmentalized neurons. *VPS35* and *VPS13C* mutations noted in PD target these mechanisms. Mutations in *GBA* comprise the most common risk factor for PD and initiate pathology by compromising lysosomal function. This is also the case for *ATP13A2* mutations. Interestingly, α -synuclein and LRRK2, key proteins involved in PD, function in different steps of the E-L pathway and target their components to induce disease pathogenesis. In this review, we discuss these E-L system genes that are linked to PD and how their dysfunction results in PD pathogenesis.

Keywords: Autophagy, Clathrin-mediated endocytosis, LRRK2, Lysosomes, Retromer complex, α -Synuclein.

J. Neurochem. (2019) **150**, 487–506.

This article is part of the Special Issue "Synuclein".

Parkinson's disease (PD) is the second most common neurodegenerative disorder, clinically identified by motor symptoms, with an increasing recognition of non-motor manifestations (Hoehn and Yahr 1967; Chaudhuri and Schapira 2009). Loss of dopaminergic neurons of the substantia nigra pars compacta (SNpc) (40–50%) and the presence of α -synuclein pathology in surviving neurons are the neuropathological hallmarks of PD. At the onset of motor symptoms, dopamine levels have decreased 60–70% in the dorsal striatum, where most SNpc dopaminergic neurons form synaptic connections (Fearnley and Lees 1991; Ma *et al.* 1997; Dickson *et al.* 2009; Kordower *et al.* 2013). Under physiological conditions, α -synuclein is predominantly present in

Abbreviations used: AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ATP13A2, ATPase cation transporting 13A2; CCVs, clathrin coated vesicles; CME, clathrin-mediated endocytosis; DCVs, dense core vesicles; E-L, endolysosomal; ESCRT, endosomal sorting complex required for transport; GAK, cyclin G associated kinase; GBA, Glucosidase Beta Acid; Gcase1, glucocerebrosidase 1; GD, Gaucher disease; GlcCer, glucosylceramide; GTPase, guanosine triphosphatase; GWAS, genome wide association study; Hsc70, heat shock cognate 70; HSP40, heat shock protein 40; HSPGs, heparan sulfate proteoglycans; ILVs, interluminal vesicles; iPSCs, induced pluripotent stem cells; KO, knockout; LAMP2a, lysosome-associated membrane glycoprotein 2; L-dopa, Levodopa; LRRK2, leucine-rich repeat kinase 2; M6P, mannose-6-phosphate; MAPK/ERK, mitogen-activated protein kinase/ extracellular signal-regulated kinase; MSNs, medium spiny neurons; MVBs, multivesicular bodies; PARK, Parkinson's disease gene; PD, Parkinson's disease; PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PINK1, PTEN Induced putative Kinase 1; PIPs, phosphoinositide phosphates; PRKN, parkin gene; RME-8, receptor mediated endocytosis 8; siRNA, small interfering ribonucleic acid; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; SNCA, alpha-synuclein gene; SNpc, substantia nigra pars compacta; SVs, synaptic vesicles; SYNJ1/ SynJ1, synaptotagmin-1; TGN, trans-golgi network; USP19, ubiquitin-specific protease 19; VPS, vacuolar protein sorting.

Received April 22, 2019; revised manuscript received July 1, 2019; accepted July 3, 2019.

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pre-synaptic terminals, aiding neurotransmitter release, whereas in PD, α -synuclein tends to aggregate and form Lewy bodies (the defining pathology of PD) (Spillantini *et al.* 1997; Spillantini *et al.* 1998). Aggregation of α -synuclein, perturbed proteostasis, mitochondrial dysfunction, oxidative stress, and impaired calcium homeostasis are the major cell-autonomous mechanisms of PD pathogenesis. Non-cell-autonomous mechanisms include cell-to-cell transmission of pathological α -synuclein and neuroinflammation (Jyothi *et al.* 2015; Poewe *et al.* 2017). So far, incomplete knowledge on causal mechanisms has limited treatment strategies to symptomatic relief. Recent studies targeting the endolysosomal (E-L) system reveal that its dysfunction can lead to the mentioned disease mechanisms, suggesting the E-L system as a key pathway in PD pathogenesis. In this review, we discuss important E-L system components, their roles in neuronal survival, and how their dysfunction causes PD.

Endolysosomal system in neurons: Custom-made for neuronal function

The E-L system comprises of complex, highly dynamic membrane-enclosed tubular-vesicular structures with overlapping properties and functions (Klumperman and Raposo 2014). They facilitate nutritional intake from a cell's microenvironment through endocytosis, neutralize pathogenic materials via phagocytosis, promote cellular proteostasis through autophagy, and maintain overall cellular homeostasis through endosomal sorting and trafficking of various proteins/enzymes across organelles (Repnik *et al.* 2013; Klumperman and Raposo 2014). E-L system functions vary greatly depending on cell type and functional state. Neurons are unique in their highly polarized intracellular compartmentalization of cell body/soma and processes including axons, axon terminals, and dendrites. This organization enables them to form complicated networks that mediate specialized functions (Fig. 1). This complexity comes at a price: burdensome demands on the E-L system, which have been increasingly implicated in neurological diseases including PD (Kett and Dauer 2016; Wang *et al.* 2018).

In neurons, synthesis of E-L components occurs primarily in the cell body/soma (Fig. 1a). These components are trafficked through the secretory pathway to their subcellular location. A prime example of this is lysosomal hydrolases trafficking. Lysosomal hydrolases are produced in the endoplasmic reticulum, then undergo glycosylation and other post-translational modifications at the Golgi apparatus [e.g. addition of mannose-6-phosphate (M6P) groups] (Nixon and Cataldo 1995). This modification directs hydrolases to lysosomes. More specifically, hydrolases form complexes with transmembrane M6P receptors, which are sequestered into clathrin-coated vesicle (CCVs) budding from the trans-Golgi networks (TGN) to form endosomes/early endosomes (Nixon and Cataldo 1995) (Fig. 1a). Inside early endosomes,

M6P receptors detach from hydrolases and are recycled back to the TGN for their next round of transport (Saftig and Klumperman 2009), while hydrolases are delivered to lysosomes (discussed below).

Simultaneously, early endosomes are also derived from the cell surface through the endocytic pathway, an integral part of the E-L system. Many of the nutrients required for neuronal function and surface receptors designated for recycling are internalized through clathrin-mediated endocytosis (CME) at the plasma membrane and delivered to early endosomes (Fig. 1a). Early endosomes derived from both the TGN and the plasma membrane mature into late endosomes, where Rab5 and Rab7 play a prominent role (Saftig and Klumperman 2009). This process occurs with the help of the endosomal sorting complex required for transport (Fig. 1a), which facilitates ubiquitin-dependent sorting of cargo/hydrolases from the outer membrane of early endosomes into interluminal vesicles (ILVs) (Wollert *et al.* 2009). Early endosomes derived from CME, if not sorted, follow a recycling pathway to the plasma membrane (Fig. 1a) (Saftig and Klumperman 2009). ILVs are enveloped inside multivesicular bodies (MVBs), which mature into late endosomes. These late endosomes deliver their luminal contents either by forming tubular extensions or by fusing with pre-existing lysosomes to form endolysosomes (Luzio *et al.* 2007). These may be considered the final compartment of the endocytic pathway, where hydrolases degrade endocytic cargo and the membranes of ILVs for reuse (Saftig and Klumperman 2009; Klumperman and Raposo 2014). In addition, there is retrograde transport of receptors (such as M6P, LAMP2a, etc) from endosomes to the TGN for reuse through the retromer complex, comprised mainly of VPS35, VPS26 and VPS29 (Saftig and Klumperman 2009) (Fig. 1a). Proteins that are meant for secretion follow a secretory pathway from the TGN (Saftig and Klumperman 2009; Klumperman and Raposo 2014). Selective capture and elimination of a cell's damaged organelles and misfolded proteins happens through a process called macroautophagy (termed hereafter as autophagy) (Ohsumi 2014), an integral part of the E-L system. Double membrane vesicles called autophagosomes encapsulate unwanted/toxic cytosolic constituents including aggregated proteins and deliver them to the lysosome for degradation (Fig. 1a) (Mizushima 2007; Kawamata *et al.* 2008). Non-selective autophagy also occurs during starvation, through which cells can recycle proteins and reuse amino acids for the synthesis of basic proteins essential for cell survival (Ohsumi 2014). Therefore, autophagy plays a pivotal role in house-keeping and maintenance of neuronal homeostasis.

Customization of the E-L system for neurons mainly happens in axons (Fig. 1b) and synapses (Fig. 1c). At pre-synaptic terminals, vesicle precursors are delivered from the cell body and undergo vesicle cycling to form functional synaptic vesicles (SVs). However, delivery of new vesicle precursors cannot match the number of SVs undergoing

exocytosis to achieve neurotransmission at an active synapse (Saheki and De Camilli 2012). This deficit is solved by highly responsive CME, recapturing fused vesicular membranes from the pre-synaptic membrane to form CCVs, which are in turn converted back to SVs through clathrin uncoating (Fig. 1c) (Heuser and Reese 1973; Morgan *et al.* 2000; Saheki and De Camilli 2012). Less understood clathrin-independent bulk endocytosis (Holt *et al.* 2003; Wu and Wu 2007) and the “kiss and run” mechanism (Fesce *et al.* 1994) are also parts of the synaptic endocytic repertoire. In addition, there are dense-core vesicles derived from the TGN that are transported down the axons. Unlike SVs, dense core vesicles do not participate in SV recycling and are primarily used to secrete long-acting neuropeptides (Nurrish 2014). While these processes are mostly confined to the pre-synaptic compartment, the E-L system's postsynaptic compartment contributes to membrane trafficking pathways primarily aimed at receptor, transporter, and ion-channel recycling (Rosendale *et al.* 2017).

Although this complex E-L system allows a neuron to function far away from its site of protein synthesis, the neuron is beholden to anterograde transport of proteins and membranes synthesized and modified in the soma for maintenance of axons and the synapses (Maday *et al.* 2014). Furthermore, constant synaptic activity, especially in continuously firing neurons like SNpc dopaminergic neurons may result in considerable build-up of damaged organelles, membranes, and misfolded proteins. These need to undergo encapsulation by an autophagosome or endosome (Fig. 1C) and retrograde transport to the soma for lysosomal degradation (Fig. 1B) (Yang *et al.* 2013). Here, long-distance axonal microtubule networks provide significant support to the neuronal E-L system (Yang *et al.* 2013). These spatial challenges to address a unique structural geometry, high metabolic demand, enhanced responsiveness to chemical/electrical stimulation, and the requirement for longevity because of their postmitotic nature, place a tremendous burden on the E-L system. Nigrostriatal dopaminergic neurons have an additional burden in terms of long and highly branched axons, larger surface area, selective transporters for external toxins, large fluctuations in free Ca^{2+} , less buffering capacity, and higher oxidative stress, reducing their threshold for degeneration (Vidyadhara *et al.* 2016; Surmeier *et al.* 2017; Vidyadhara *et al.* 2017; Vidyadhara *et al.* 2019). These vulnerabilities are further exacerbated with aging. Faced with environmental and genetic risk factors, disease pathogenesis abounds.

E-L system dysfunction: the holy grail of PD pathophysiology?

In 1997, identification of dominant mutations in the α -synuclein gene (*SNCA*, *PARK1*) which encodes α -synuclein as a cause of familial PD (Polymeropoulos *et al.* 1997),

brought a conceptual shift toward the importance of genetics in disease pathogenesis. Subsequent findings revealed various other genetic mutations, with at least 10–15% of PD patients having familial PD of an absolute genetic origin. Leucine-rich repeat kinase 2 (*LRRK2*, *PARK8*) and Parkin (*PRKN*, *PARK2*) mutations are the most common genetic variants of dominant and recessively inherited PD respectively (Deng *et al.* 2018). Mutations and gene multiplications of *SNCA* (*PARK1* and *PARK4*) (Singleton *et al.* 2003; Chartier-Harlin *et al.* 2004) and Glucosidase Beta Acid (*GBA*) are also highly prevalent, whereas PD pathology was also noted with abnormal forms of Parkinsonism associated with deglycase or DJ1 (*PARK7*), and PTEN induced putative kinase 1 (*PINK1*, *PARK6*) (Deng *et al.* 2018). There is also compelling evidence for a genetic component to sporadic PD. In fact, a rigid distinction between the genetic and environmental basis of PD pathogenesis may not exist considering that cases are phenotypically indistinguishable (except for the onset) (Baba *et al.* 2006), implying genetic and sporadic cases share common underlying mechanisms. Even after considerable studies on these familial mutations and their interaction with environment, the core pathomechanisms of PD are still unclear, limiting treatment strategies. Over the past 10 years, advancements in high-throughput sequencing, the application of genome wide association studies (GWAS), and meta-analysis, have identified new genetic mutations (refer to Deng *et al.* 2018 for a list of all PARK genes and risk alleles). Intriguingly, most of these identified genes have links to the E-L system (refer Table 1). Mechanistic studies also suggest that these proteins are the downstream targets of previously identified broad spectrum dysfunction in α -synuclein and *LRRK2* mutations. It is now becoming clear that most PD-linked genes (Table 1) and known pathomechanisms are, in one way or another, associated with the E-L system, strongly suggesting this pathway as the key master regulator of PD pathogenesis. Recent studies suggest that neurodegeneration is initiated at the dopaminergic pre-synaptic terminals of striatum, years before the clinical diagnosis (Imbriani *et al.* 2018). Evidence that most of the E-L system genes associated with PD function at this terminal further establishes their fundamental role in PD pathogenesis. Below, we discuss our understanding of the functions and dysfunctions of these PD-linked E-L system genes. We also discuss how other key PD-associated proteins target E-L pathways.

Clathrin-mediated synaptic vesicle endocytosis: A prime target?

SV endocytosis is predominantly mediated through CME (Fig. 1c). Following exocytosis, the SV membrane is endocytosed primarily through CME, which involves formation of a clathrin coat, composed of an outer clathrin layer, and an inner layer of adaptors (Saheki and De Camilli 2012). The most common adaptors, such as AP-2 and AP180, bind

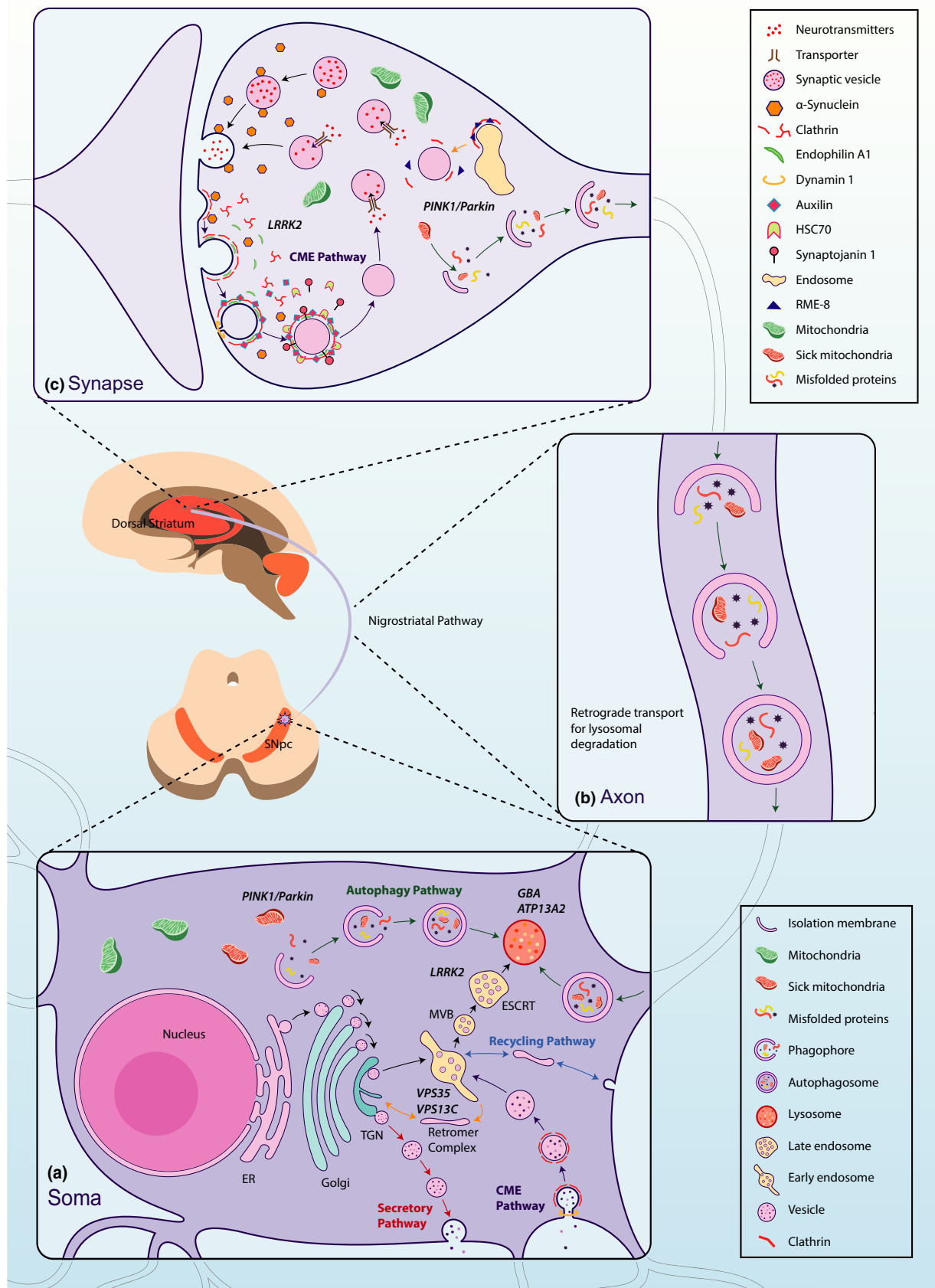


Fig. 1 E-L system in the compartments of a nigrostriatal dopaminergic neuron, with the normal functional locations of E-L system proteins linked to PD. (a) Soma: E-L system proteins that form structural and functional components are synthesized in the neuronal soma. Following production in the endoplasmic reticulum (ER) and post-translational modifications in the Golgi apparatus, proteins (e.g. lysosomal hydrolases) are packed into vesicles and delivered to the trans-Golgi network (TGN). In the TGN, based on the nature of post-translational modification, the vesicles are diverted either toward lysosomes (black arrows) or to the secretory pathway (red arrows). Vesicles that are destined for lysosomes bud off from the TGN to form early endosomes. Simultaneously, early endosomes carrying nutrients and surface receptors for recycling are also derived from the plasma membrane through clathrin mediated endocytosis (CME, purple arrows). These early endosomes, with the help of the endosomal sorting complex required for transport (ESCRT), pass through highly dynamic tubulovesicular structures like multivesicular bodies (MVB) and/or late endosomes before fusing with lysosomes. Leucine-rich repeat kinase 2 (LRRK2) plays a significant role in this sorting, whereas Glucocerebrosidase 1 (GBA/Gcase 1) and ATPase cation transporting 13A2 (ATP13A2) are crucial for normal lysosomal function. Early endosomes from the cell surface that are not sorted follow the recycling pathway back to the plasma membrane (blue arrows). Retrograde transport of receptors that are used for sorting from early endosomes to TGN occurs through the retromer complex where Vacuolar Protein Sorting 35 (VPS35) and VPS13C take part (yellow arrows). An expanding membrane sac called a phagophore sequesters malfunctioning organelles (e.g. sick mitochondria) and misfolded proteins to form a double-membraned autophagosomes. These later

fuse with lysosomes and release contents for degradation and recycling of components; the complete process is called autophagy (green arrows). PTEN Induced Putative Kinase 1 (PINK1) and Parkin are important in identifying sick mitochondria and diverting them to a specialized form of autophagy called mitophagy. (b) Axon: Axons form a crucial bridge for retrograde transport of endosomes/autophagosomes from pre-synaptic terminals to soma for lysosomal degradation. A gradual development of phagophore to full-fledged autophagosome happens within the axon (green arrows) as the encapsulated cargo arrives towards the proximal end. (c) Synapse: Neurotransmitter release in the pre-synaptic terminal is regulated principally by the synaptic vesicle (SV) cycle, an integral part of the neuronal E-L system. Neurotransmitters are loaded into these SVs through specialized transporters. Upon arrival of an action potential in the synaptic terminal, these SVs fuse with the plasma membrane at active sites and release neurotransmitters via exocytosis (black arrows). Empty SVs are then recycled through CME (Purple arrows). α -Synuclein and endophilin A play roles here in membrane bending and SV curvature formation. Clathrin forms a layer on these invaginations with the help of adaptors, and in conjunction with dynamin leads to membrane fission forming clathrin coated vesicles (CCV). Clathrin uncoating is a prerequisite for SV recycling, which is performed by coordinated action of auxilin, Hsc70, endophilin A1, and synaptojanin 1. Phosphorylation by LRRK2 is necessary for activation of these proteins. Endosomes derived from the plasma membrane also form SVs in the nerve terminal where RME-8 plays a role in clathrin sorting (yellow arrow). Synaptic autophagy and mitophagy are also crucial in maintaining the health of the pre-synaptic terminal (green arrows), where PINK1/Parkin are crucial.

Table 1 PD genes linked to E-L system with their PARK nomenclature, proteins they encode, and their primary function and original reference of their discovery.

PD genes that are linked to E-L system				
PARK	Gene	Protein	Function	Reference
PARK1	<i>SNCA</i>	α -Synuclein	Synaptic vesicle cycling	Polymeropoulos <i>et al.</i> (1997)
PARK2	<i>PRKN</i>	Parkin	E3 Ubiquitin ligase	Mori <i>et al.</i> (1998) Kitada <i>et al.</i> , (1998)
PARK4	<i>SNCA</i>	α -Synuclein	Synaptic vesicle cycling	Singleton <i>et al.</i> , (2003) Chartier-Harlin <i>et al.</i> , (2004)
PARK6	<i>PINK1</i>	PINK1	Serine/Threonine kinase	Valente <i>et al.</i> , (2004)
PARK8	<i>LRRK2</i>	LRRK2	Kinase/GTPase	Paisán-Ruiz <i>et al.</i> , (2004); Zimprich <i>et al.</i> , (2004)
PARK9	<i>ATP13A2</i>	ATPase type 13A2	ATPase	Ramirez <i>et al.</i> , (2006); Di Fonzo <i>et al.</i> , (2007)
PARK16	<i>RAB29</i>	Ras-Related Protein Rab-7L1	Rab GTPase	Satake <i>et al.</i> (2009)
PARK17	<i>VPS35</i>	VPS35	Retromer	Wider <i>et al.</i> , (2008) Vilarinho-Güell <i>et al.</i> , (2011)
PARK19	<i>DNAJC6</i>	Auxilin	Co-chaperone	Edvardson <i>et al.</i> , (2012) Köroğlu <i>et al.</i> , 2013
PARK20	<i>SYNJ1</i>	Synaptojanin-1	Polyinositol phosphatase	Krebs <i>et al.</i> , (2013) Quadri <i>et al.</i> , (2013)
PARK21	<i>DNAJC13</i>	RME-8	Co-chaperone	Vilarinho-Güell <i>et al.</i> , (2014)
PARK23	<i>VPS13C</i>	VPS13C	Retromer, Lipid transporter	Lesange <i>et al.</i> , (2014), Schormair <i>et al.</i> , (2018)
Risk Genes	<i>GBA</i>	Glucocerebrosidase 1	Glucosidase	Sidransky <i>et al.</i> , (2009)
	<i>GAK</i>	Cyclin-G Associated Kinase	Co-chaperone	Nalls <i>et al.</i> , (2014) Chang <i>et al.</i> , (2017)
	<i>SH3GL2</i>	Endophilin A1	Membrane bending protein	Nalls <i>et al.</i> , (2014) Chang <i>et al.</i> , (2017)

to endocytic motifs on the SV membrane, as well as phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] present in the plasma membrane. Endophilin A1 and α -synuclein drive membrane bending and curvature formation of the developing clathrin coated pit (Bai *et al.* 2010; Westphal and

Chandra 2013). Following this, the clathrin coated pit in conjunction with dynamin and other accessory proteins undergoes membrane fission to form a CCV (Saheki and De Camilli 2012). Regeneration of SVs from CCVs and recycling of clathrin and related coat components for further

endocytosis requires un-coating of clathrin from CCVs (Fig. 1c). The clathrin uncoating step is carried out by the coordinated action of the PI phosphatase synaptojanin-1 and the co-chaperone auxilin with Hsc70. Endophilin A1 and cyclin G Associated Kinase (GAK) also regulate clathrin uncoating to facilitate CME at the pre-synaptic terminal (Fig. 1c) (Saheki and De Camilli 2012). Mutations of these E-L system genes in familial PD suggest synaptic endocytosis dysfunction as a prime trigger for PD pathogenesis.

The DNAJ family of heat shock proteins in the pre-synaptic terminal and beyond

Auxilin

DNAJC6 gene encodes auxilin, the major pre-synaptic endocytic protein linked to PD. Auxilin is a brain specific co-chaperone belonging to the DNAJC family (Table 1), a subclass of heat shock proteins (HSP40). Auxilin plays an important role in clathrin un-coating, recruiting ATP-activated Hsc70 to CCVs, which in turn, disassembles clathrin from vesicles facilitating their recycling (Lemmon 2001; Gorenberg and Chandra 2017) (Fig. 1c). Loss-of-function autosomal recessive mutations of auxilin (*PARK19*) have been shown to cause juvenile/early onset PD, first observed in two teenage brothers of a Palestinian family (Edvardson *et al.* 2012). The study noted abnormal mRNA splicing and a decrease in auxilin mRNA levels. Other variants of auxilin mutations were later noted in a Brazilian family with similar mis-splicing of mRNA, whereas mutations affecting the J-domain (p.Q791*, p.Q846* and p.R927G) were observed in a few other consanguineous families (Koroglu *et al.* 2013; Olgiati *et al.* 2016). Along with typical PD symptoms, patients with nonsense mutations have cognitive impairment, one of the cardinal non-motor symptoms of PD. Epilepsy and mental retardation have also been noted for some PD patients with auxilin mutations (Koroglu *et al.* 2013). These human genetic studies suggest widespread neuronal dysfunction besides nigrostriatal dopaminergic neurodegeneration. Hence, future studies on post-mortem brains documenting PD-typical Lewy body pathology and dopaminergic neuronal loss are necessary to concretely categorize the pathology as PD or parkinsonism.

One of the few preclinical studies characterizing *DNAJC6* reveals that auxilin down-regulation leads to progressive dopaminergic neuronal loss and motor disabilities in *Drosophila*. Furthermore, loss of auxilin exacerbates α -synuclein over-expression and paraquat (an environmental toxin)-mediated dopaminergic loss, thus aggravating previously established phenotypes in fly models of PD (Song *et al.* 2017). Interestingly, the brains (deep cerebellar nuclei) of adult homozygous auxilin knockout (KO) mice revealed an increased number of CCVs and empty cages at the synapse. Similar defects were noted in primary cortical and hippocampal neurons accompanied by defective SV endocytosis

(Yim *et al.* 2010). The impairment was not immediately detrimental to the neurons, which may explain the post-developmental onset of PD symptoms and slow disease progression. Evaluating whether similar disruptions occur in nigrostriatal dopaminergic neurons and how it mediates pathogenesis will prove crucial in understanding the role of auxilin and the E-L system in PD. An initial step toward this suggests that LRRK2 phosphorylates auxilin, attenuating auxilin's normal function in human iPSC-derived dopaminergic neurons. The study further revealed that phosphorylation of auxilin by over-expressing LRRK2 leads to accumulation of oxidized dopamine and α -synuclein over-expression, pathological hallmarks of PD (Nguyen and Krainc 2018). Whether loss-of-function mutations in auxilin independently exert pathology through the same mechanisms is unknown. However, this newfound link between LRRK2 and auxilin points to the crucial and widespread involvement of E-L system in PD pathogenesis.

GAK

GAK is another DNAJC family protein (*DNAJC26*/Auxilin 2), homologous (~50%) to auxilin but expressed throughout the body (Lemmon 2001). In a meta-analysis of PD GWAS across 13,708 patients and 95,282 controls (Nalls *et al.* 2014), GAK was identified as a risk gene for PD which was confirmed in another recent largescale study (Chang *et al.* 2017) (Table 1). However, GAK's role in PD is still contentious because of presence of mutations in *TMEM175* in a nearby independent locus, which shares the same promoter as GAK. What adds credence is that, like auxilin, GAK functions in clathrin un-coating by recruiting Hsc70 to CCVs (Lemmon 2001) (Fig. 1C). Results show that GAK over-expression can partially compensate for loss of auxilin, and is also noted to up-regulate naturally in auxilin-KO mice (Yim *et al.* 2010). Another similarity between auxilin and GAK has been noted in their interaction with LRRK2 (Jinn *et al.* 2017; Nguyen and Krainc 2018). Brain-specific loss of GAK and ubiquitous KO of GAK are both lethal (postnatal and embryonic respectively) (Lee *et al.* 2008a). The inability of auxilin to compensate for GAK in these mice suggests another unique role for GAK in neurons, perhaps a significant one in dopaminergic neurons. Further *TMEM175* deficiency is noted to cause PD-like α -synuclein aggregation, along with impairment of lysosomal and mitochondrial function (Jinn *et al.* 2017). Clarity on the role of GAK will prove to be crucial not only in establishing its involvement in PD but also in consolidating CME as the prime target in PD pathogenesis.

RME-8

Receptor mediated endocytosis 8 (RME-8) or *DNAJC13* is another DNAJC family co-chaperone and principally functions in regulating retromer and clathrin un-coating dynamics on endosomes (Gorenberg and Chandra 2017) (Fig. 1C).

DNAJC13 mutations (p.N855S) in autosomal-dominant PD (*PARK21*) noted first in a Dutch-German-Russian family of Mennonite origin, with multiple incidences (Table 1) (Vilarino-Guell *et al.* 2014; Gustavsson *et al.* 2015). Post-mortem brains of PD patients with *DNAJC13* mutations revealed strong immunoreactivity for Lewy bodies in the substantia nigra, along with brain areas implicated in non-motor symptoms of PD. Nigral Lewy bodies were also found to be co-localized with RME-8 (Vilarino-Guell *et al.* 2014). Unlike other DNAJC proteins implicated in PD, *in vitro* studies have revealed that the *DNAJC13* mutation confers toxic gain-of-function, leading to E-L cargo trafficking deficits (Norris *et al.* 2017). The *DNAJC13* mutation is also predicted to affect overall endosomal trafficking, even in the soma and postsynaptic compartment (Vilarino-Guell *et al.* 2014), unlike the synapse-specific dysfunction predicted for auxilin. Recently, the link between *DNAJC13* and PD has been muddled by an independent analysis of the same Mennonite family which did not find *DNAJC13* mutations. Instead, all the affected individuals were identified to carry mutations in *TMEM230* (Deng *et al.* 2016), which also functions in E-L system, perhaps at synapses. Hence, further studies are warranted to confirm the genetic link between *DNAJC13* and PD. Still, evaluating the RME-8/*DNAJC13* mediated E-L system dysfunction might provide valuable insights in understanding PD.

Synaptojanin-1 and endophilin A: Partners in crime?

Two other major synaptic endocytic proteins besides the DNAJ family that are implicated in PD are synaptojanin-1 (SYNJ1) and endophilin A. SYNJ1 is a Sac domain-containing protein with two different phosphatase domains; 5-phosphatase and Sac1, which target different phosphoinositide phosphates (PIPs) (McPherson *et al.* 1996). 5-phosphatase regulates SV endocytosis by dephosphorylating PI(4,5)P₂ to facilitate uncoating of CCVs in cooperation with Hsc70 and auxilin (Fig. 1c), whereas Sac1 domain has gained importance because of its link to PD (*PARK20*, Table 1) (Cremona *et al.* 1999; Mani *et al.* 2007; Saheki and De Camilli 2012). The p.R258Q missense mutations in the Sac domain were identified independently in two consanguineous families of Italian and Iranian origin. Homozygous missense mutations of *SYNJ1* Sac domain are noted to cause autosomal recessive PD with extrapyramidal features identical to patients with auxilin mutations (Krebs *et al.* 2013; Quadri *et al.* 2013; Olgiati *et al.* 2014). Interestingly, they responded poorly to L-dopa treatment, indicating the need for preclinical studies to understand the exact pathomechanisms. Recent observations by Cao *et al.*, suggest that the Sac domain plays a previously unappreciated role in clathrin uncoating. Mice with *SynJ1* Sac mutations (knock-in) developed PD-like characteristics and featured synaptic endocytosis defects, including accumulating of CCVs. Furthermore, elevated

auxilin and Parkin levels were noted in these mice, suggesting the profound effect of Sac mutations on other proteins linked to PD (Cao *et al.* 2017). Studies on fibroblasts from patients with *SYNJ1* mutations suggested defective endocytic trafficking (Quadri *et al.* 2013). Other studies have shed light on the involvement of SynJ1 in regulating synaptic autophagy. Mutations in the Sac1 domain of *SynJ1* in both *Drosophila* neurons and *PARK20* patient-derived iPSCs severely affected synaptic autophagosome formation but not endocytosis (Vanhouwaert *et al.* 2017). This discrepancy and the precise relationship between SV endocytosis and synaptic autophagy remains to be clarified. However, the *Drosophila* model revealed loss of dopaminergic neurons, providing evidence for the contribution of synaptic autophagy defects in pathogenesis of PD (Vanhouwaert *et al.* 2017). Considering its dual enzymatic activity, SynJ1 is also believed to play a role in fast endophilin-mediated, clathrin-independent endocytosis which is an alternative to CME (Watanabe *et al.* 2018) and several other processes. It remains to be determined if SYNJ1 exert its effects on PD pathogenesis through any of the alternative endocytic pathways.

SYNJ1 recruitment to endocytic membrane is facilitated by endophilin A (Fig. 1c), a protein drawing attention in PD pathogenesis, despite its locus variation only being identified as a risk factor (Table 1) (Chang *et al.* 2017). This is because of endophilin A's close association with SYNJ1 (Song and Zinsmaier 2003; Cao *et al.* 2017) and its link to most important proteins involved in PD. One of our earlier studies indicated that over-expression of α -synuclein decreases endophilin A levels, while deletion of synucleins had the opposite effect (Westphal and Chandra 2013). As noted in *SynJ1* knock-ins, endophilin KO also leads to synaptic vesicle endocytosis defects, accumulation of CCVs, elevation of Parkin levels, and other parkinsonian features (Milosevic *et al.* 2011; Cao *et al.* 2014). Gain-of-function mutations in LRRK2 hyper-phosphorylate endophilin A, the fly homolog, leading to synaptic endocytosis dysfunction (Matta *et al.* 2012). LRRK2-mediated phosphorylation of endophilin A is also noted to regulate synaptic autophagy (Soukup *et al.* 2016). Owing to the relatively higher frequency of LRRK2 mutations and its involvement in sporadic PD, endophilin A may prove to be another crucial protein involved in PD.

Autophagy in PD; cause and effect, hand in hand

Neurons need to constantly maintain homeostasis by preserving healthy organelles and functional proteins, while selectively capturing and eliminating damaged organelles and misfolded proteins. Autophagy, an integral component of the E-L system, is an intracellular proteostasis pathway that performs this housekeeping function (Mizushima *et al.* 1998;

Nixon 2013; Suresh *et al.* 2018b) (Fig. 1A, 1). It was initially identified in cell lines to function in maintaining energy homeostasis during critical periods of development and in response to nutrient depletion (Mizushima 2007). Intriguingly, nutrient depletion does not induce autophagy in neurons, but a complete lack of autophagy can cause lethal neurodegeneration, emphasizing its important role in survival (Hara *et al.* 2006; Komatsu *et al.* 2006). Since neurons are post-mitotic and long-lived, they are particularly vulnerable to accumulation of toxic proteins (Nixon 2013). In addition, greater dependency of neurons on mitochondria for energy instead of glycolytic pathways demands constant mitochondrial turnover (Kann and Kovacs 2007), leading to many malfunctioning mitochondria. Both these factors play major roles in the pathogenesis of PD.

PINK1 and Parkin: Role in mitophagy and beyond

Under physiological conditions, autophagy plays a crucial role in clearing unwanted proteins, as well as damaged mitochondria through a targeted mechanism called mitophagy (Nguyen *et al.* 2016). Loss-of-function mutations in *PINK1* and *Parkin* (*PARK2* and *PARK6*), result in recessively inherited early onset PD and significantly, both genes were discovered to be essential for mitophagy (Kitada *et al.* 1998; Mori *et al.* 1998; Valente *et al.* 2004). *PINK1* is a serine/threonine protein kinase that acts as a surveillance molecule for mitochondrial health and accumulates on the outer membrane of abnormal mitochondria (Nguyen *et al.* 2016). *PINK1* attracts recruitment of *Parkin* to damaged mitochondria, which through its E3 ubiquitin ligase activity (Narendra *et al.* 2010; Kane *et al.* 2014), coats the mitochondria with ubiquitin triggering autophagosomes to engulf the mitochondria (Narendra *et al.* 2008), culminating in lysosomal degradation (Fig. 1a1). Discoveries of *PINK1* and *Parkin* mutations were the first to shed light on the direct involvement of autophagy and E-L system in PD pathogenesis (Pickrell and Youle 2015). These observations also helped in validating previous neurotoxic studies (Przedborski *et al.* 2004) which identified mitochondrial dysfunction as one of the primary causes of neurodegeneration. Though the symptoms are indistinguishable from sporadic cases, several studies on brains of PD patients with *Parkin* mutations do not show α -synuclein aggregation and Lewy body pathology (Doherty and Hardy 2013). Only one report suggests *PINK1*-linked parkinsonism to be associated with Lewy pathology (Samaranch *et al.* 2010). This prompts us to speculate if there are alternative neuropathological cascades in PD that are α -synuclein independent but centered around the E-L system.

In addition to their well-defined roles in mitophagy, *Parkin* was identified to modulate E-L system through Rab7 ubiquitination (Song *et al.* 2016), whereas *PINK1* phosphorylates some Rab proteins (Lai *et al.* 2015). It is also worth noting that several of these Rab proteins (for example Rab7) regulate mitochondrial dynamics and quality control independent of

Parkin/PINK1 (Wang *et al.*, 2018). *Parkin* mutations are also known to directly affect retromer recruitment (Williams *et al.* 2018) and interact strongly with VPS35 by ubiquitinating it (Malik *et al.* 2015; Martinez *et al.* 2017). *Parkin* binds to endophilin A and functions in its ubiquitination (Cao *et al.* 2014). *Parkin* also ubiquitinates EPS15 to regulate epidermal growth factor receptors and phosphoinositide 3-kinase (PI(3)K)-Akt signaling that plays a role in neuronal survival (Fallon *et al.* 2006). These developments suggest mitophagy-independent routes these proteins may take toward PD. Further investigations of *Parkin* and *PINK1* in this direction might provide crucial insights in understanding neurodegenerative mechanisms that are Lewy-body independent.

Synaptic autophagy and axonal transport: a road less travelled in PD research

α -Synuclein aggregates initially in pre-synaptic termini, where the protein is enriched (Kramer and Schulz-Schaeffer 2007). α -Synuclein aggregates are a major target for autophagy-mediated degradation (Xilouri *et al.* 2008). In both genetic and sporadic PD, aggregated α -synuclein is likely to enhance autophagosome formation to meet the higher demand for its degradation (Wong and Holzbaur 2015). Once threshold reaches beyond compensatory limits, mutant/over-expressed α -synuclein starts to accumulate, turning detrimental to cellular homeostasis. In fact, A53T and A30P α -synuclein mutants blocked their own uptake and other substrates by lysosomes for degradation through the chaperone-mediated autophagy pathway *in vitro* (Cuervo *et al.* 2004). Dopamine-modified α -synuclein is identified to further exacerbate this process, which may explain the selective vulnerability of dopaminergic neurons in PD (Martinez-Vicente *et al.* 2008). A recent study on mammalian cells and transgenic mice shows that α -synuclein over-expression mediated autophagy defects are exerted through Rab1a inhibition (Winslow *et al.* 2010). Further understanding of the mechanisms for α -synuclein-autophagy crosstalk in PD might have crucial therapeutic applications. Backing this notion, both *in vitro* and *in vivo* studies show that enhancing autophagy and autophagosome-lysosome fusion could ameliorate PD-like features by clearing aggregated α -synuclein (Suresh *et al.* 2017; Suresh *et al.* 2018a).

Several other PD causing genes associated with the E-L system exert their pathological effects through impairing synaptic autophagy. The R258Q SynJ1 mutation directly affects autophagosome biogenesis and maturation at synaptic terminals of flies and neurites of human iPSC-derived neurons. The observation of dopaminergic neuronal loss in these flies suggests autophagosome generation defects as one of the possible mechanisms through which SynJ1 mutations lead to dopaminergic neurodegeneration and PD (Vanhouwaert *et al.* 2017). Pre-synaptic endocytic membrane bending protein endophilin A also has functions in providing docking sites for autophagy proteins and facilitating autophagosome expansion.

This is achieved by phosphorylation of the endophilin A-BAR domain by LRRK2, as noted in a fly model (Soukup and Verstreken 2017). Congruent to these observations, mice lacking endophilin A revealed deficiency in autophagosome formation and developed a parkinsonian phenotype (Murdoch *et al.* 2016). These studies, along with recent finding of clathrin involvement in autophagy regulation (Rong *et al.* 2012), suggest a cooperative cross-talk between SV endocytosis and autophagy.

Macromolecules and organelles in the pre-synaptic terminal travel a long distance from the soma, making them old and vulnerable. Rapid turnover of proteins, membranes, receptors, and vesicles in the pre-synapse assigns autophagy a major role in maintaining synaptic homeostasis. This tasks synaptic autophagy with quality control responsibilities. Though there is compelling evidence that compromised autophagy contributes to PD, these studies primarily looked at the soma from a proteinopathy or mitophagy perspective (Lynch-Day *et al.* 2012). Selective inactivation of autophagy by conditional knockout of autophagy protein atg7 in dopaminergic neurons leads to enhanced dopamine release and rapid pre-synaptic recovery with abnormal axonal profiles (Hernandez *et al.* 2012). Conversely, autophagy enhancement through mTOR inhibition slows dopamine release, reduces the number of synaptic vesicles, and increases autophagosome density in the axons (Hernandez *et al.* 2012). These results emphasize the importance of synaptic autophagy in normal dopamine transmission and further evaluation is required to understand if additional synaptic autophagy defects exist, which may lead to PD pathogenesis.

Another unique and stressful situation for neurons is that autophagosomes loaded with unwanted materials from an active pre-synaptic terminal, need to be transported a long distance to the soma for lysosomal degradation (Fig. 1b). Microtubule and scaffolding proteins aid in this retrograde axonal transport of autophagosomes and are crucial for the normal functioning of E-L system (Birdsall and Waites 2019). During their transport, autophagosomes mature into autolysosomes by fusing with late-endosomes/lysosomes towards the proximal end of axon (Nikolietopoulou and Tavernarakis 2018). There are recent studies suggesting lysosomal degradation, especially of mitophagosome mediated by Parkin-PINK1, occurs at the distal end. This is predicted to provide rapid neuroprotection against oxidative stress by damaged mitochondria, without requirement for retrograde transport (Ashrafi *et al.* 2014; Ashrafi and Schwarz 2015). Axonal transport aids this function through anterograde transport of E-L system components required for degradation from soma towards the distal end. Any defect in this to-and-fro transport machinery can completely clog the E-L system and other processes. Considering its functional significance in neuronal survival, axonal transport might hold clues for understanding pathological mechanisms. Abnormal

axonal swelling with lysosomal accumulation has been noted in Alzheimer's disease, blocking the retrograde transport of autophagosomes (Nixon *et al.* 2005; Gowrishankar *et al.* 2015). Similar understanding on the health of axonal transport machinery and its dynamics in PD is crucial toward finding commonalities amongst neurodegenerative diseases.

Sometimes, there are traffic issues: E-L system trafficking in PD

The multi-compartmentalized nature of neurons puts tremendous pressure on the endosomal sorting and trafficking machinery, which is also true in case of nigrostriatal neurons. The vacuolar protein sorting (VPS) group of proteins that form retromer complex comprising of VPS35, VPS26, and VPS29 are crucial in retrograde trafficking and sorting of protein cargos from endosomes to the TGN, facilitating their recycle and reuse (Fig. 1a) (Seaman *et al.* 1998; Haft *et al.* 2000). The retromer complex also functions in sorting proteins/cargo to regulate endosomal and lysosomal maturation (Vagnozzi and Pratico 2018). Pedigrees with single allelic mutation in *VPS35* revealed autosomal dominant, late-onset, L-dopa-responsive PD, with striking similarities to idiopathic PD (Wider *et al.* 2008; Vilarino-Guell *et al.* 2011). Broadly, it is proposed that the mutant *VPS35* fails to associate with other components of the retromer complex, resulting in impaired retrograde trafficking (Williams *et al.* 2017). Induced loss-of-function mutations of *VPS35* *in vitro* resulted in enlargement of late-endosomes and aggregation of α -synuclein oligomers (Follett *et al.* 2016). Another study on flies revealed ceramide accumulation because of *VPS35* deficiency, suggesting lysosomal dysfunction (Lin *et al.* 2018). α -Synuclein accumulation in nigral dopaminergic neurons, loss of dopaminergic neurons, reduced dopamine transporter levels in the striatum, and locomotory deficits were noted in *VPS35*-deficient mice (Tang *et al.* 2015a; Ishizu *et al.* 2016). Further *in vitro* studies of *VPS35* (D620N) mutant cells revealed dysfunction in endosome-to-Golgi retrieval of lysosomal membrane proteins (LAMP2a) and accelerated lysosomal degradation of LAMP2a (Tang *et al.* 2015a). LAMP2a is considered important in directing misfolded α -synuclein to lysosomes, suggesting a mechanism for α -synuclein accumulation in *VPS35* deficiency (Tang *et al.* 2015a). *VPS35* mutations also affect autophagy and dendritic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor trafficking (Zavodszky *et al.* 2014; Munsie *et al.* 2015). *VPS35* mutations bring severe abnormality in mitochondrial dynamics, as seen in mouse dopaminergic neuronal cultures and in PD patient derived fibroblasts (Tang *et al.* 2015b). Oxidative stress implicated in sporadic PD might mediate pathogenesis through *VPS35*-driven mitochondrial defects (Wang *et al.* 2016). *VPS35* also has a role in biogenesis of mitochondria-derived vesicles,

further suggesting that its dysfunction causes mitochondrial pathology and neurodegeneration (Sugiura *et al.* 2014). VPS35 is functionally linked to LRRK2, boosting its kinase activity (Mir *et al.* 2018). In fact, decreased VPS35 levels were noted in the SNpc of PD patients with *LRRK2* mutations (Zhao *et al.* 2018). Interestingly, over-expression of VPS35 ameliorated locomotor deficits and enhanced lifespan in *LRRK2* mutant models (MacLeod *et al.* 2013; Linhart *et al.* 2014), which was also seen in *Parkin* mutant models (Malik *et al.* 2015). Neuroprotection was also noted against MPP⁺ induced toxicity in dopaminergic neurons (Bi *et al.* 2013). Along with its E-L sorting functions, these links between VPS35 and α -synuclein, LRRK2, Parkin, lysosomal dysfunction, autophagy, mitochondrial perturbations, and oxidative stress strongly suggest VPS35 as a good therapeutic target.

Another VPS family member associated with PD is VPS13C. Five allelic variations in the *VPS13C* gene can lead to autosomal recessive, early-onset PD, primarily because of premature termination of VPS13C (Lesage *et al.* 2016; Schormair *et al.* 2018). Patients were noted with cardinal PD symptoms and extrapyramidal signs. Post-mortem studies revealed α -synuclein/ubiquitin positive-Lewy bodies in the brainstem, limbic system, hippocampus, and cortical associative areas (Lesage *et al.* 2016). A few studies reveal that VPS13 functions in the TGN-endosomal cycle (De *et al.* 2017), whereas recent discoveries have also located it on the outer mitochondrial membrane (Lesage *et al.* 2016). In fact, siRNA-mediated silencing of VPS13C resulted in lower mitochondrial membrane potential, mitochondrial fragmentation, increased respiratory rates, exacerbated Parkin-dependent mitophagy, and up-regulation of Parkin in response to mitochondrial damage (Lesage *et al.* 2016). A new study has revealed VPS13C's role in forming membrane contact sites and facilitating lipid transfer between the ER and mitochondria (Kumar *et al.* 2018). This is noteworthy, considering lipid transfer's increasing recognition in disease pathogenesis.

Lysosomes: PD may also begin at the end of the tunnel

Since lysosomes are the destination for most E-L system vesicles carrying a variety of cargos for degradation and recycling (Fig. 1a), dysfunction in any of its components results in lysosomal storage disorders, severely perturbing cellular homeostasis. A growing body of evidence suggests lysosomal storage disorders increase risk for PD pathogenesis (Robak *et al.* 2017). Among them, Gaucher disease (GD) confers the greatest risk, where 5–15% of GD patients or carriers develop PD later in life, to an extent where causal *GBA* mutations are the most common genetic risk factor for PD (Table 1) (Aharon-Peretz *et al.* 2004; Simon-Sanchez *et al.* 2009; Bultron *et al.* 2010). *GBA* encodes glucocerebrosidase 1 (Gcase1), which facilitates breakdown of glucosylceramide (GlcCer) into

glucose and ceramide. In its absence, GlcCer accumulates and undergoes partial enzymatic deacylation to glucosylsphingosine and other more complex glycosphingolipids, which mediate disease pathogenesis. *GBA* is a relatively well-studied E-L system gene with regards to PD, in terms of post-mortem brains, clinical investigations, and basic research. Reduced Gcase1 activity in substantia nigra, putamen, and other brain areas of PD patients with *GBA* mutations have been noted along with GlcCer and glycosphingolipids accumulation (Gegg *et al.* 2012; Alcalay *et al.* 2015). Interestingly, similar observations were made even in sporadic PD patients (Alcalay *et al.* 2015; Rocha *et al.* 2015), suggesting an even broader role for *GBA* than just mutations and potentially involve epigenetic and environmental modifiers of *GBA*. Gcase1 levels decrease with aging, the major risk factor for PD (Rocha *et al.* 2015; Hallett *et al.* 2018), indicating *GBA*'s possible role in lowering the nigral neurons' threshold to develop PD. Cognitive areas such as hippocampus, frontal cortex, and amygdala also showed relative decrease in Gcase1 activity, along with a significant elevation of hippocampal glucosylsphingosine in sporadic cases (Gegg *et al.* 2012). This correlates with a higher Lewy body burden identified in the hippocampus and medial temporal regions, as well as in a diffused cortical Lewy body pathology in *GBA*-linked PD (Watson and Leverenz 2010; Hallett *et al.* 2018). These pathological observations strengthen clinical findings that cognitive deterioration is severe in PD patients with *GBA* mutations compared to that of sporadic cases (Liu *et al.* 2016; Mata *et al.* 2016). These features indicate that mouse models of PD with *GBA* mutations are important in studying lipid dyshomeostasis and mechanisms of cognitive dysfunction in PD.

A recent study by Taguchi *et al.*, provides evidence for an association between loss of Gcase1 activity, glycosphingolipids accumulation, and α -synuclein aggregation, both *in vitro* and *in vivo* models of *Gba* mutation (Taguchi *et al.* 2017). Glucosylsphingosine which promoted pathological α -synuclein aggregates, capable for further self-templating, was shown to accumulate in the brains of young homozygous *Gba* mutant and KO mice. This strongly implicates glucosylsphingosine in PD pathogenesis. Even in heterozygous *Gba* mutations, the Gcase1 activity decreased with age to an extent where mice reached threshold for haploinsufficiency, mimicking the human condition. Furthermore, the *Gba* mutant mice, when crossed with well-characterized α -synuclein transgenic PD mice, revealed accelerated PD progression including α -synuclein aggregation and motor deficits (Taguchi *et al.* 2017). α -Synuclein aggregation has also been seen in human iPSCs-derived neurons from GD and PD patients that are exposed to Gcase1 inhibitors, and in those carrying *GBA* mutations (Zunke *et al.* 2018). Heterozygous mutations in *GBA* also cause misprocessing of Gcase1 in the ER, leading to ER retention of Gcase1. This produces ER stress and auto-lysosomal perturbations causing an abnormal lipid profile and increased α -synuclein

accumulation and secretion (Ron and Horowitz 2005; Fernandes *et al.* 2016). This study along with a few other observations suggest dopaminergic dysfunction because of gain-of-function mutations in *Gba* (Cullen *et al.* 2011; Sidransky and Lopez 2012; Fernandes *et al.* 2016). However, therapeutic strategies based on loss-of-function observations have been successful, arguing against a gain-of-function model. Activation of residual Gcase1 by small molecules and chaperones and ameliorating glycosphingolipid accumulation by inhibiting GlcCer synthase lowered α -synuclein pathology and reinstated lysosomal health (Richter *et al.* 2014; Sardi *et al.* 2017; Charvin *et al.* 2018). These strategies are already being tested in clinics (Richter *et al.* 2014).

ATPase cation transporting 13A2 (ATP13A2), another lysosomal protein which localizes on its membrane (Fig. 1a) with unknown function, has been implicated in PD (*PARK9*, Table 1). Autosomal recessive, early-onset parkinsonism because of *ATP13A2* mutations first noted in Kufor-Rakeb Syndrome, followed by recent studies in other forms of parkinsonism (Ramirez *et al.* 2006; Di Fonzo *et al.* 2007; Park *et al.* 2011; Eiberg *et al.* 2012). Fibroblasts from these patients showed impaired lysosomal acidification and proteostasis, leading to inefficiency in substrate degradation and autophagosome clearance. These observations were replicated in *ATP13A2* knockdown dopaminergic cell lines, where its restoration re-established lysosomal homeostasis and ameliorated cell death (Dehay *et al.* 2012). Down-regulation of *ATP13A2* was also seen in nigral neurons of sporadic PD patients, along with *ATP13A2* traces in Lewy bodies (Dehay *et al.* 2012). A loss-of-function *ATP13A2* mutant mouse model revealed age-related lysosomal dysfunction, protein aggregation, and gliosis leading to motor abnormalities. Interestingly, the levels of total and oligomeric α -synuclein was normal, despite ubiquitinated protein aggregation, cathepsin-D dysfunction, and autophagy defects (Kett *et al.* 2015). These observations suggest that *ATP13A2* mutations may lead to PD through an α -synuclein independent mechanism.

E-L system; a key target of key PD-associated proteins?

α -Synuclein

Misfolded α -synuclein can trigger PD pathogenesis in the pre-synaptic terminals of nigrostriatal neurons (Kramer and Schulz-Schaeffer 2007; Spinelli *et al.* 2014; Imbriani *et al.* 2018). This may well be one of the earliest pathogenic events in PD, where α -synuclein aggregates target SV endocytosis, SV trafficking, and autophagy components of E-L system to initiate or exacerbate dopaminergic neuronal dysfunction.

α -Synuclein and other family members function in pre-synaptic terminals, where they modulate SV distribution and neurotransmission (Greten-Harrison *et al.* 2010; Burre 2015;

Vargas *et al.* 2017) (Fig. 1c). The biophysical properties of synucleins reveal their ability to sense and modify membrane curvature (Middleton and Rhoades 2010; Shen *et al.*, 2012; Westphal and Chandra 2013). These functions suggest that synucleins regulate SV exocytosis and endocytosis (Greten-Harrison *et al.* 2010; Vargas *et al.* 2014). Studies in KO mice in fact show that synucleins normally function post SV exocytosis, in fusion pore expansion and/or control the kinetics of SV endocytosis by facilitating membrane curvature (Vargas *et al.* 2014; Lautenschlager *et al.* 2017; Logan *et al.* 2017). α -Synuclein also appears to control SV availability for release by regulating SV clustering (Diao *et al.* 2013; Vargas *et al.* 2017).

On a pathological level, several studies show that over-expression of α -synuclein inhibits neurotransmission. The locus of this consistent effect is not clear. Studies have shown that elevated α -synuclein levels and oligomeric α -synuclein inhibit SV exocytosis in a SNARE-dependent manner or by affecting SV clustering (Nemani *et al.* 2010; Huang *et al.* 2019). On the other hand, considerable evidence suggests that aggregated α -synuclein primarily impacts SV endocytosis, which is of relevance to E-L system genes linked to PD. In lamprey giant synapses, injection of aggregated α -synuclein inhibit clathrin uncoating as evidenced by the accumulation of CCVs when SV recycling was evoked by high-frequency stimulation (Busch *et al.* 2014). A recent study also suggests that extra synuclein might sequester Hsc70, thus affecting clathrin uncoating and subsequently causing SV recycling defects (Banks *et al.*, 2019). A similar observation was made in mammalian Calyx of Held synapse, where acute elevation of monomeric and mutant α -synuclein levels decreased SV endocytosis with a frequency-dependent effect on SV exocytosis. This was also seen upon transgenic expression of α -synuclein (Xu *et al.* 2016; Eguchi *et al.* 2017). Over-expression of mutant α -synuclein also affects dopamine transporter trafficking on the pre-synaptic membrane, leading to dopamine dyshomeostasis (Kisos *et al.* 2014). The observations that over-expression and deletion of synucleins have similar effects on SV exo- and endocytosis (Lautenschlager *et al.* 2017) suggest that SV cycling is exquisitely sensitive to α -synuclein levels. Further understanding of the precise mechanisms for interactions of α -synuclein and synaptic E-L system proteins involved in PD will provide crucial insights on early causal pathogenic events in PD.

At the soma, aggregated α -synuclein mainly targets E-L system trafficking, especially the retromer pathway. Recent studies propose that aggregated α -synuclein disrupts the retromer complex by interacting through VPS26 or VPS29, and thus preventing the recruitment of adaptors like Snx3 and SNX-BAR dimers (Chung *et al.* 2017; Patel and Witt 2018). Deficiency of VPS35 in dopaminergic neurons impairs endosome-to-Golgi retrieval of receptors like LAMP2a,

enhancing its lysosomal degradation. LAMP2a is a critical receptor for chaperone-mediated autophagy of misfolded α -synuclein, so its deficiency leads to a vicious cycle of α -synuclein accumulation (Tang *et al.* 2015a), which might be the case in patients with VPS35 mutations. Over-expression of α -synuclein also directly compromises macroautophagy through inhibition of Rab1a (Winslow *et al.* 2010). It is also pertinent to note that aggregated α -synuclein in the soma is a key target of treatment strategies in development through induction of macroautophagy (Suresh *et al.* 2017; Suresh *et al.* 2018a).

Recent developments suggest that α -synuclein pathology spreads in a prion-like manner through synaptically connected regions in a manner consistent with Braak staging (Braak *et al.* 2003; Luk *et al.* 2012). The E-L system plays a prominent role in cell-to-cell transmission of preformed α -synuclein filaments, leading to the spread of phospho- α -synuclein pathology. Internalization of fibrillar α -synuclein occurs via endocytosis (as well as tunnelling nanotubules) and follows the neuronal endosomal pathway to lysosomal degradation (Rodriguez *et al.* 2018). Heparan sulfate proteoglycans, lymphocyte-activation gene 3, $\alpha 3$ isoform of Na^+/K^+ ATPase, and a few endocytic vesicle receptors are predicted to act as receptors for α -synuclein preformed fibrils (Holmes *et al.* 2013; Shrivastava *et al.* 2015; Mao *et al.* 2016; Ihse *et al.* 2017). *In vitro* studies suggest that internalized α -synuclein fibrils are localized in both early endosomes and late endolysosomes (Lee *et al.* 2008b; Karpowicz *et al.* 2017). The lipid bilayer of endosomes restricts cytoplasmic spillage, so most endocytosed α -synuclein is degraded by lysosomes. How a fraction of α -synuclein preformed fibrils escapes this barrier and goes on to initiate seeding still remains unclear (Bieri *et al.* 2018). Studies of α -synuclein axonal transport predict that retrograde transport of α -synuclein preformed fibrils is mediated by endocytic vesicles and is twice as fast and more efficient than anterograde transport (Brahic *et al.* 2016). This strengthens the notion that PD pathology spreads retrogradely from the synaptic terminal to soma. The E-L system is also involved in exocytosis of fibrillized α -synuclein, facilitating its prion-like spread. An ER-bound enzyme with chaperone activity USP19 (ubiquitin-specific protease 19) has been shown to deliver misfolded α -synuclein to late endosomes, which then fuses with the plasma membrane through a unique process called misfolding-associated protein secretion (Lee *et al.* 2016). Another recent study suggests that the DNAJ/Hsc70 complex could facilitate α -synuclein exocytosis (Fontaine *et al.* 2016). Over-expression of *ATP13A2* and *GBA* have been shown to increase exocytosis of α -synuclein (Tsunemi *et al.* 2014; Fernandes *et al.* 2016). Together, these observations identify the E-L system as the primary conduit for the spread of α -synuclein-mediated pathology in PD.

LRRK2

Leucine-Rich Repeat Kinase 2 (LRRK2) is a multidomain large protein with both GTPase and kinase activity along with other domains for protein-protein interactions (Kett and Dauer 2012). Epidemiological, histopathological, and clinical investigations have all established *LRRK2* gene mutations as the most common genetic cause of PD (*PARK8*, Table 1) (Paisan-Ruiz *et al.* 2004; Zimprich *et al.* 2004), with a considerable contribution to sporadic cases (Kett and Dauer 2012). Over eight identified *LRRK2* mutations cause PD. The mutation at G2019S is the most common and is known to increase LRRK2 kinase activity (West *et al.* 2005; Smith *et al.* 2006; Kett and Dauer 2012). LRRK2 functions have been explored through PD-related studies, which have revealed its close association with the E-L system. LRRK2's role in vesicular trafficking, lysosomal homeostasis, and autophagy are well-accepted. Phosphorylation by LRRK2 is essential for a subset of RAB GTPases' function of E-L vesicular sorting and trafficking (Steger *et al.*, 2016; Steger *et al.*, 2017). In an over-expression system, RAB7L1 was noted to interact strongly with LRRK2 at the switch-II site (Steger *et al.*, 2017), unlike as seen in *in vitro* kinase assays (Steger *et al.*, 2016). Several *LRRK2* mutations significantly affect RAB pathways, where variations in Rab7L1's interactions with LRRK2 have been identified to increase the risk of sporadic PD. In fact, recent GWAS identify *RAB29* encoding RAB7L1 (*PARK16*, Table 1) as an independent risk gene for PD (Satake *et al.* 2009). Rab7L1 up-regulation could attenuate LRRK2-induced toxicity in dopaminergic neurons (MacLeod *et al.* 2013). This is predicted to be functioning through VPS35 and VPS26 (Linhart *et al.* 2014). VPS35 also cooperates with LRRK2 to regulate SV recycling and dopaminergic synaptic release (Inoshita *et al.* 2017), thus suggesting a prominent role for the retromer complex in LRRK2-mediated PD pathogenesis.

LRRK2 regulates lysosomal protein trafficking and morphology (Kuwahara *et al.* 2016; Eguchi *et al.* 2018). In *LRRK2* mutants, perinuclear lysosomal clustering was noted, mediated by increased phosphorylation of RAB7 (Hockey *et al.* 2015). Two-pore channels are the calcium channels present on acidic vesicles of the E-L system and help in regulating their trafficking and fusion of lysosomes with autophagosomes/endosomes (Calcraft *et al.* 2009). Abnormal lysosomal morphology and density seen in fibroblasts from patients with LRRK2 mutations could be reversed by inhibiting these two-pore channels (Hockey *et al.* 2015). Thus, these channels might be one potential mediator of E-L system dysfunction in LRRK2-associated PD. Lysosome-related proteins involved in PD such as Gcase1, LAMP2A, and ATP13A2 are also down-regulated in PD patients with *LRRK2* mutations (Zhao *et al.* 2018), suggesting that lysosomes are one of LRRK2's prime targets.

Mechanisms for LRRK2-mediated autophagy defects are still elusive. The ability of LRRK2 kinase inhibitors to reverse autophagy dysfunction suggest that kinase activity may be significant (Manzoni *et al.* 2013; Saez-Atienzar *et al.* 2014). Mutant *LRRK2* is also a substrate for chaperone-mediated autophagy, impairment of which could exacerbate pathological implications (Orenstein *et al.* 2013). Accumulation of MVBs and malformed autophagosomes were noted in *LRRK2* R1441C mutations (Alegre-Abarrategui *et al.* 2009). Intra-axonal autophagic vacuole defects in nigral dopaminergic projections were seen in mice expressing human *LRRK2* (Li *et al.* 2009; Tagliaferro *et al.* 2015). *LRRK2* also regulates synaptic autophagy through phosphorylation of endophilin A (Soukup *et al.* 2016). G2019S mutations are known to induce autophagy through MAPK/ERK kinases (Bravo-San Pedro *et al.* 2012; Bravo-San Pedro *et al.* 2013), and some studies suggest that *LRRK2*-mediated autophagy regulation is largely independent of mTOR (Manzoni *et al.* 2013). Since RAB-mediated trafficking is crucial for autophagy, which is also affected in *LRRK2* mutations, it is important to evaluate if autophagy defects are the consequences of RAB dysregulation or a direct effect of *LRRK2* mutations.

LRRK2 regulates the SV cycle by interacting with E-L system proteins that are also implicated in PD: auxilin, SYNJ1, and endophilin A. *LRRK2* overactivity phosphorylates auxilin at its clathrin binding domain, making their interaction weaker. This leads to disruption of SV endocytosis (Nguyen and Krainc 2018). This was seen in patient iPSC-derived dopaminergic neurons, along with accumulation of oxidized dopamine, increased α -synuclein levels, and decreased Gcase1 activity (Nguyen and Krainc 2018). Hyperphosphorylation of endophilin A and synJ1 by *LRRK2* significantly altered synaptic endocytosis (Matta *et al.* 2012; Islam *et al.* 2016; Pan *et al.* 2017). A recent study showed that *LRRK2* can directly bind to clathrin-light chains through its GTPase domain, affecting Rac1-mediated endosomal dynamics (Schreij *et al.* 2015). *LRRK2* also perturbs synaptic exocytosis through phosphorylation of N-ethylmaleimide-Sensitive Factor which up-regulates SNARE disassembly (Belluzzi *et al.* 2016). Furthermore, *LRRK2* is highly expressed in dorsal striatal medium spiny neurons (MSNs), the post-synaptic compartment of nigrostriatal pathway. G2019S mutation of *LRRK2* leads to abnormally high activity of MSNs and morphological perturbations during development (Matikainen-Ankney *et al.* 2016). Abnormal synaptogenesis and dopamine receptor activation in the developing MSNs mediated by dysfunctional protein kinase A was seen in *LRRK2* R1441C mutation (Parisiadou *et al.* 2014). These defects can permanently modify the circuit structure and function, predisposing the individuals to both motor and non-motor dysfunctions of PD. *LRRK2* mutation-mediated endocytosis defects were noted predominantly in these MSNs, along with nigral dopaminergic

neurons and not in cortical or hippocampal neurons (Pan *et al.* 2017; Maas *et al.* 2017). This suggests a heightened vulnerability in the E-L system of nigrostriatal circuit for *LRRK2* mutations.

Increased *LRRK2* kinase activity mediated elevation of exocytosis may also be relevant for cell-to-cell transfer of misfolded α -synuclein (Lin *et al.* 2009; Bae *et al.* 2018; Schapansky *et al.* 2018). *LRRK2* can be selectively recruited to overloaded lysosomes and divert undegraded materials out of the kidney cells, along with secretion of α -synuclein from endosomes (Eguchi *et al.* 2018). Studies also demonstrate heightened uptake of α -synuclein fibrils through endocytosis in the presence of pathogenic *LRRK2* (Bae *et al.* 2018). However, *LRRK2*'s role in prion-like spreading of α -synuclein remains speculative, until confirmation that similar mechanisms exist in neurons.

Future directions

Clinical description and diagnosis of PD has changed little since James Parkinson's characterization of the disease 200 years ago (Parkinson 2002). In the 1960s, identification of dopamine deficiency as the cause of motor abnormalities led to symptomatic relief for patients (Carlsson *et al.* 1957). Although some success has been noted with deep brain stimulation (Limousin *et al.* 1998), we are still relying on symptomatic relief. The last three decades of basic research have enriched our understanding of PD's cellular and molecular mechanisms, bringing tangible hope for disease modifying treatment strategies. Identification of risk genes and conceptual shifts in our understanding through genetic models point to E-L system dysfunction as the key mediator of PD pathogenesis. Further strengthening this concept, PD pathogenesis appears to begin at synaptic terminals where endo- and exocytotic machinery is most active and where α -synuclein and *LRRK2* exert their effects through the E-L system and vice-versa. Studies so far suggest that altered flux through the E-L system in nigral dopaminergic neurons that have lesser threshold for degeneration is the key trigger for neurodegeneration. Moving forward, critical elucidation of the mechanisms behind sorting, trafficking, and recycling defects demands detailed studies of auxilin and other DNAJ family proteins, along with SYNJ1, endophilin A, and the retromer complex. Studying these proteins/genes will also provide crucial insights into less explored roles of synaptic autophagy, axonal transport, and clathrin-independent endocytosis in PD. Recent surveys of PD patients note preservation of cognitive functioning as a major unmet need. Further investigation of *GBA* mutations implicated in PD is a promising avenue considering *GBA*'s known involvement in cognitive disorders like Dementia with Lewy Bodies. Along with *GBA*, studying *ATP13A2* and other lysosomal genes will enrich our understanding of lysosomal dysfunction, potentially bringing us closer to repurposing drugs used

in lysosomal storage disorders for PD. Affirming ongoing clinical trials targeting autophagy, α -synuclein, and LRRK2, an enhanced understanding of E-L system-mediated pathogenesis will prove crucial in developing novel treatment strategies and diagnostic tools for PD.

Acknowledgments and conflict of interest disclosure

We thank lab members for fruitful discussions on this review topic and important inputs to the figure. This work was supported by NIH (R01NS110354, R01NS083846), Nina Compagnon Hirshfield Parkinson's Disease Research Fund and DOD (W81XWH-17-1-0564). VDJ is supported by DOD CDMRP Early Investigator Research Award (W81XWH-19-1-0264). The authors have no conflict of interest to declare.

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