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14. ABSTRACT

Parkinson's disease (PD) is a debilitating neurodegenerative disorder characterized by both motor and cognitive symptoms and the formation of Lewy body inclusions containing aggregated alpha-synuclein protein. Growing evidence suggests that alpha-synuclein aggregation may originate in the gastrointestinal tract and spread to the central nervous system. This 'gut-to-brain' hypothesis of PD suggests that environmental toxin exposures may contribute to the initial formation of alpha-synuclein aggregates in the gut. However, it remains unknown (1) the effects of gut-derived alpha-synuclein on cognitive ability, (2) the mechanisms by which alpha-synuclein spreads, and (3) how novel therapies can target alpha-synuclein induced cognitive decline. In the reporting period, we have completed Specific Aim 1, which is to determine the effects of gut-derived alpha-synuclein on learning and memory cognitive function. Our results showed that gut-derived alpha-synuclein significantly impairs short-term associative learning & memory in our new C. elegans models of PD, recapitulating key cognitive symptoms in PD. The expression levels of the major learning regulator, casy-1, were reduced by PFF treatment, though there is no direct interaction of PFFs with CASY-1 in vivo, suggesting an indirect mechanism by which PFFs disrupt this learning regulator. We are now conducting Specific Aim 2, which is to uncover the mechanisms by which gut-derived alpha-synuclein enters neurons to affect cognition. We are on track with the timeline in the approved Statement of Work, and anticipate that in the next reporting period we will complete Specific Aim 2, and Specific Aim 3, which is to identify novel treatments for alpha-synuclein-induced cognitive dysfunction by targeting cellular metabolism. The results of this study will provide one of the first analyses of how gut-derived alpha-synuclein affects cognition, offer new targets for halting the toxic spread of alpha-synuclein from gut to brain, and identify new potential therapies for cognitive decline in PD.

15. SUBJECT TERMS

Parkinson's disease, alpha-synuclein, cognition, gut-to-brain, C. elegans

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1. INTRODUCTION:

Parkinson's disease (PD) is a devastating neurodegenerative disorder in which patients experience motor and cognitive symptoms. The protein α-synuclein is linked to the disorder both genetically and pathologically, yet the role of α-synuclein in the disease remains unclear. Toxic α-synuclein proteins may first appear in neurons of the gastrointestinal tract, and then spread to the central nervous system. This 'gut-to-brain' hypothesis of PD is consistent with environmental exposures that may initiate the disease process in the gut. The purpose of the funded research is to determine the effects of gut-derived α-synuclein on cognitive ability, the mechanisms by which α-synuclein spreads, and to identify novel therapies that can target α-synuclein-induced cognitive decline. To accomplish this, we are using the small nematode worm, *Caenorhabditis elegans* (*C. elegans*), which allows for rapid, systematic testing of potential disease mechanisms and treatments. The scope of the funded project is to use established learning & memory assays to test cognitive function in our new *C. elegans* PD models fed exogenous α-synuclein pre-formed fibrils (PFFs), screen highly conserved genes to determine if they facilitate α-synuclein spreading in these models, and test FDA-approved drugs and dietary supplements to identify those that may improve cognitive function in PD. This work will provide one of the first accounts of how 'gut-to-brain' spreading of α-synuclein affects cognition, and may offer new targets and potential therapies for halting this neurotoxic spread, thereby advancing the fields of neurotoxin exposure and treatment-related Parkinson's research. Moreover, the funded project is also meant to establish the PI in these fields, through highly productive mentoring by the Co-Mentors, and presentation of the results to the larger scientific community in the form of peer-reviewed publications and oral presentations.

2. KEYWORDS:

Parkinson's disease, alpha-synuclein, cognition, gut-to-brain, *C. elegans*

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Determine the effects of gut-derived α-synuclein on learning and memory cognitive function.

Major Task 1: Conduct learning & memory assays in C. elegans model of neurotoxic α-synuclein exposure
Milestone(s) Achieved: Characterize learning & memory phenotypes including learning, short- and long-term memory, and forgetting, in C. elegans fed neurotoxic α-synuclein pre-formed fibrils (PFFs).
Timeline (Months): 1-6.
Percentage of Completion: 100%.

Major Task 2: Identify which learning & memory regulators mediate α-synuclein exposure neurotoxicity
Milestone(s) Achieved: Identify key learning & memory factors that mediate the effects of gut-derived α-synuclein on cognition, and whether there is a direct interaction between α-synuclein and these factors
Timeline (Months): 6-10.
Percentage of Completion: 100%.

Specific Aim 2: Uncover the mechanisms by which gut-derived α-synuclein enters neurons to affect cognition

Major Task 3: Screen 17 cell surface glycoprotein receptors/regulators for those that mediate short-term memory deficits in C. elegans model of neurotoxic α-synuclein exposure
Milestone(s) Achieved: Identify glycoprotein receptors and/or regulators that mediate the effects of gut-derived α-synuclein on cognition, and begin establishing my professional footprint in neurotoxin exposure Parkinson's research by presenting my findings at a national conference
Timeline (Months): 11-14.
Percentage of Completion: 33%. (As per the approved SOW, this Major Task is underway/ in-progress at the time of submitting this Annual Report.)

Major Task 4: Determine if α-synuclein interacts with cell surface glycoprotein receptors and/or pathway components
Milestone(s) Achieved: Identify whether there is a direct interaction between α-synuclein and cell surface glycoprotein receptors/regulators that mediate α-synuclein neurotoxicity, publication of results from Aims 1 and 2 in peer-reviewed journals
Timeline (Months): 15-18.
Percentage of Completion: 0%. (As per the approved SOW, this Major Task has not yet begun at the time of submitting this Annual Report.)

Specific Aim 3: Identify novel treatments for α-synuclein-induced cognitive dysfunction by targeting cellular metabolism

Major Task 5: Screen 14 compounds for those that improve short-term memory function in C. elegans model of neurotoxic α-synuclein exposure
Milestone(s) Achieved: Identify potential therapeutics that restore cognitive function in C. elegans α-synuclein neurotoxicity model, and further establish my professional footprint in treatment-based Parkinson's research by presenting my findings at a national conference
Timeline (Months): 17-21.
Percentage of Completion: 0%. (As per the approved SOW, this Major Task has not yet begun at the time of submitting this Annual Report.)

Major Task 6: Test the effects of FDA-approved drugs and dietary supplements on mitochondrial function
Milestone(s) Achieved: Determine the effects of gut-derived α-synuclein on mitochondrial function and potential therapeutics that restore normal mitochondrial activity, publication of results from Aim 3 in peer-reviewed journal
Timeline (Months): 22-24.
Percentage of Completion: 0%. (As per the approved SOW, this Major Task has not yet begun at the time of submitting this Annual Report.)

What was accomplished under these goals?

Specific Aim 1: Determine the effects of gut-derived α-synuclein on learning and memory cognitive function.

Major Task 1: Conduct learning & memory assays in *C. elegans* model of neurotoxic α-synuclein exposure

Subtask 1: Test learning & short-term memory using positive olfactory assays

Subtask 2: Test learning & long-term memory using positive olfactory assays

For Specific Aim 1 Major Task 1, our major activity has been to conduct learning & memory assays in the *C. elegans* models. In Subtask 1, the specific objective was to test short-term memory, and in Subtask 2, the specific objective was to test long-term memory.

Significant results and key outcomes:

We are very excited to report that **α-synuclein PFF exposure in *C. elegans* causes significant loss of short-term learning and memory function (Figure 1)**, recapitulating key cognitive symptoms in PD. These assays are performed as follows: Worms expressing

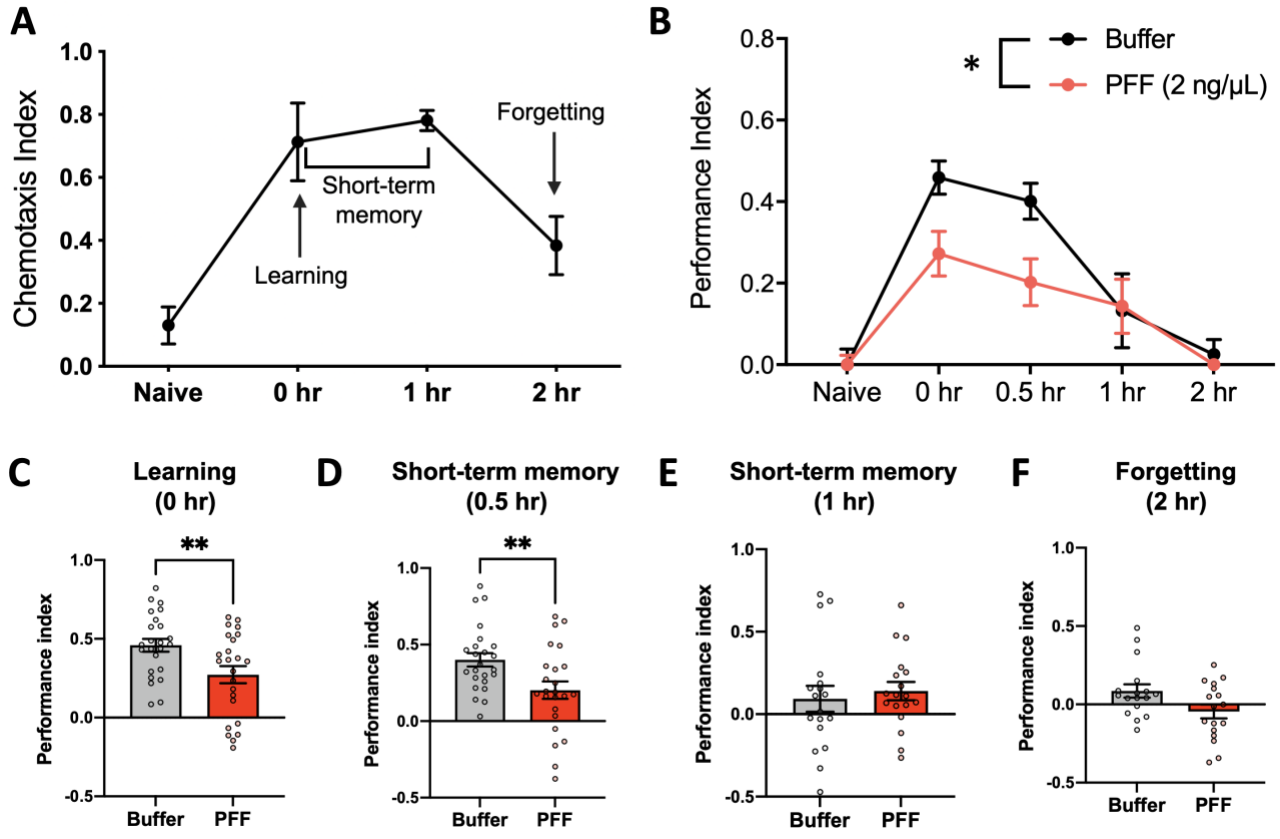
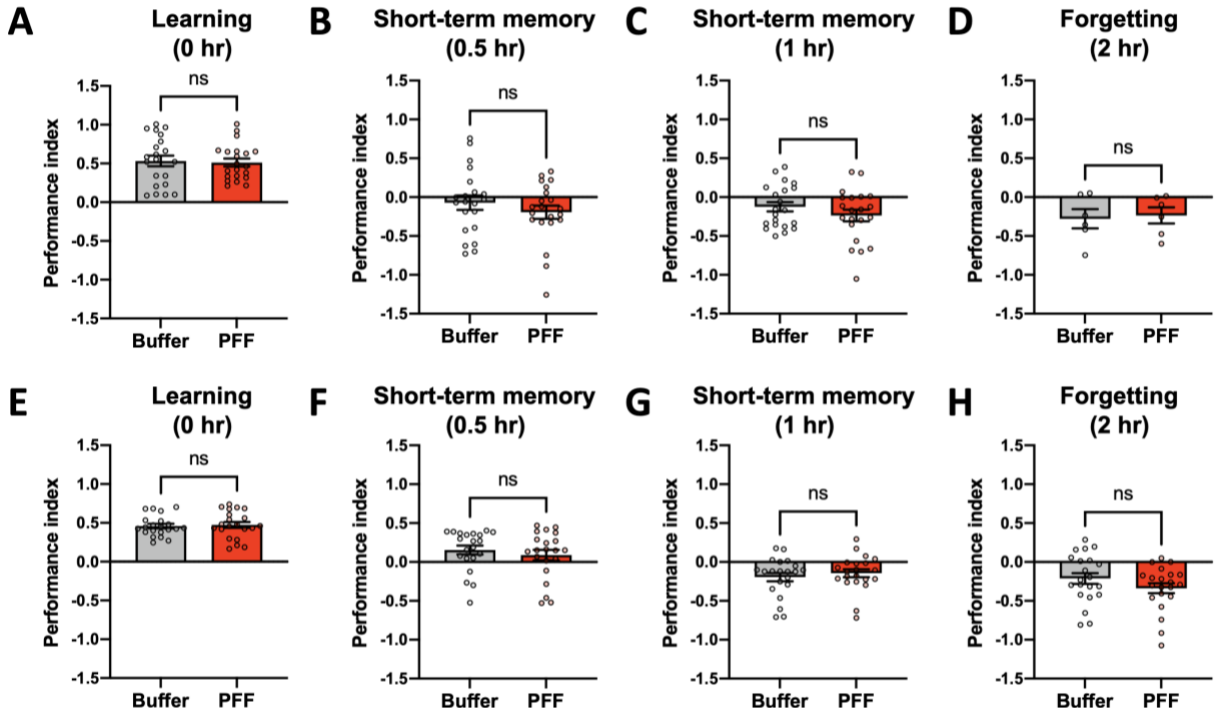


Figure 1: Learning & memory assays in *C. elegans* PD models fed neurotoxic α-synuclein PFFs reveal significant cognitive deficits on day 4 of adulthood. (A) Example of learning & memory in non-transgenic *C. elegans*. **(B)** PFF-fed worms (strain UM0011) perform significantly worse than controls in short-term memory assays. **(C-F)** Closer examination of the different phases of learning & memory reveals specifically a learning defect **(C)** and short-term memory defect at 0.5 hr post-training for the PFF group. Each data-point represents a replicate with 30-100 worms.

pan-neuronal human α-synuclein are fed an exogenous source of human α-synuclein PFFs in order to stimulate the prion-like spreading of disease from gut-to-brain, in accordance with our previously published observations[1]. PFF feeding is performed for 24 hours starting on day 1 of adulthood, in order to preclude any effects on normal development. Thereafter, the worms are tested for learning & memory ability using established protocols[2]. Briefly, on the day of the short-term memory assay, the worms are starved for 1 hr and then exposed for 1 hr to a pairing of food and the odorant butanone ('training phase'), during which the worms develop an association between the odorant and food. At multiple time-points following training, the learned preference of the worms for the odorant is tested by placing 30-100 worms onto a petri dish plate in which the worms can then choose to move towards the odorant or a control solvent solution. The number of worms that moved (chemotaxed) towards each is then counted, and the chemotaxis index per plate indicates the worms' preference for the odorant. A value of 0 indicates no preference, whereas a value of 1 shows that 100% of the worms preferred the odorant. When 2 or more groups are being compared, chemotaxis index values for each group are normalized to the pre-training ('naïve') value, and this is expressed as performance index. Worms with intact learning & memory abilities show high preference for the odorant that diminishes over the span of 2 hrs **(Figure 1A)**.

We have found that **PFF-fed worms have significant defects in both learning, and short-term memory at 0.5 hrs post-training (Figure 1B-D)**, **whereas later phases of short-term memory and forgetting are unaffected (Figure 1E-F)**. Moreover, these **short-term learning and memory defects are age-dependent (Figure 2)**, as shown by intact learning



<u>Figure 2: Age-dependence of learning & memory deficits</u>. *C. elegans* (strain UM0011) fed PFFs were tested for learning & memory function on day 2 **(A-D)** and day 3 **(E-H)**, revealing intact cognition preceding the day 4 deficits observed in Figure 1. Each data-point has 30-100 worms.

& memory function on day 2 **(Figure 2A-D)** and day 3 **(Figure 2E-H)**. The emergence of cognitive dysfunction only on day 4 and not earlier is consistent with the age-association of human PD. Therefore, **these results further validate the use of our *C. elegans* neurotoxic α-synuclein models to study cognitive decline in PD.**

Importantly, the **short-term learning and memory deficits on day 4 are dependent on prion-like mechanisms (Figure 3)**, consistent with the hypothesized prion-like spreading of α-synuclein from gut-to-brain in PD. As expected, we found that the exogenous α-synuclein must be aggregated to have neurotoxic effects, since feeding worms human α-synuclein monomer (at the same concentration) did not produce either the learning defect **(Figure 3A)** or short-term memory defect **(Figure 3B)**. In addition, the toxicity of PFFs requires host α-synuclein, since PFF feeding in a worm strain that lacks human α-synuclein expression also had intact learning **(Figure 3C)** and memory **(Figure 3D)** on day 4.

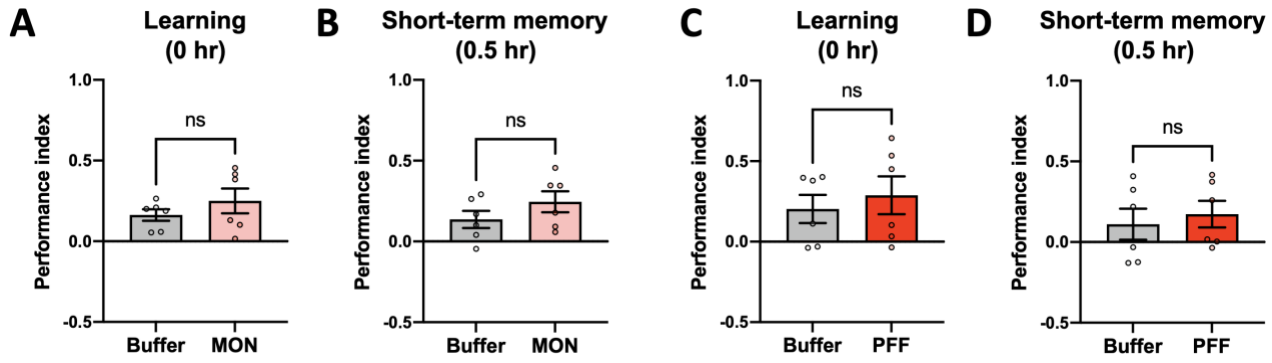


Figure 3: Learning & memory defects in *C. elegans* PD models are dependent on prion-like mechanisms. (A-B) Feeding *C. elegans* (strain UM0011) α-synuclein monomer (MON) instead of PFFs is insufficient to produce either learning or memory deficits on day 4. **(C-D)** Feeding PFFs to worms that lack pan-neuronal human α-synuclein expression (strain BY250) is also insufficient to produce either learning or memory deficits on day 4. Each data-point has 30-100 worms.

While the short-term learning and memory assays were performed successfully and led to the important findings highlighted in Figures 1-3, unfortunately we were not able to successfully perform long-term memory assays. The difference between the assays is the number of training sessions in which the odorant is paired with food: for the worms to develop a short-term memory, only 1 training session is necessary, which we reliably and consistently achieve. However, for the worms to develop a long-term memory, the assay requires 7 consecutive training sessions[2]. The long-term memory assay is therefore considerably more difficult to perform. Despite multiple attempts, consultation with the lab that created these assays (Dr. Coleen Murphy at Princeton University, with whom I did my postdoc), and extensive guidance from my Co-Mentors, we were unable to achieve reliable long-term memory in the controls (non-transgenic worms). Rather than continuing to devote time and resources towards more troubleshooting, with the support of my Co-Mentors, we decided to focus on the exciting short-term memory data, achieving the Major Task 1 Milestones of Characterizing learning & memory phenotypes including learning, short-term memory, and forgetting, in *C. elegans* fed neurotoxic α-synuclein PFFs.

Other achievements:
In the process of adapting the published *C. elegans* learning & memory protocols[2] to new protocols that can be performed with α-synuclein treatments, **we have been able to prepare a manuscript detailing these new methods** (please see Appendix). We anticipate submission of this manuscript to a peer-reviewed journal by the end of summer 2023. This publication will not only help to guide other labs who are using our PFF *C. elegans* models to study PD, but will continue to advance my career in the field of PD neurotoxin research.

Major Task 2: Identify which learning & memory regulators mediate α-synuclein exposure neurotoxicity

Subtask 1: Measure expression levels of key learning & memory regulators by qPCR and Western blot, and (if applicable) phosphorylation state by Western blot
Subtask 2: Generate fluorescent reporter *C. elegans* strains for learning & memory regulators altered in task 2.1
Subtask 3: Test for interaction of α-synuclein with learning & memory regulators altered in task 2.1 using confocal imaging of strains generated in task 2.2 and using *in vitro* binding assays

For Specific Aim 1 Major Task 2, our major activity has been to test known learning & memory regulators for their potential role in PFF-induced short-term memory impairment. In Subtask 1, the specific objective was to measure the levels of these regulators, in Subtask 2, the specific objective was to generate strains to further study any regulators whose expression levels were found to be altered, and in Subtask 3, the specific objective was to test for a potential interaction of α-synuclein with those regulators found to be altered.

Significant results and key outcomes:
Given that in Major Task 1 we found a significant impairment in learning and short-term (0.5 hr) memory, we further investigated in Major Task 2 whether known regulators of these learning & memory phases have altered expression levels that may underlie the observed cognitive deficits. Previous studies have identified that the learning phase is regulated by the genes *casy-1*, *mut-7*, *hen-1*, and *glr-1*, whereas the 0.5 hr memory phase is regulated by the genes *acy-1* and *nos-1*[3]. We performed real-time quantitative PCR (qPCR) to measure transcript levels of these genes in the PD worms fed α-synuclein PFFs as well as buffer-treated controls **(Figure 4)**. In terms of learning regulators **(Figure 4A-C)**, we detected a significant decrease in *casy-1* expression in the PFF-treated worms on day 4 **(Figure 4A)**, which may potentially explain the learning deficit observed at the same timepoint **(Figure 1C)**. Expression levels of the learning regulators *mut-7* **(Figure 4B)** and *hen-1* **(Figure 4C)** were not significantly altered by PFF treatment, and *glr-1* levels were not detected despite using two different primer sets. In terms of 0.5 hr memory regulators, there was no change in the expression of *nos-1* in PFF-fed animals **(Figure 4D)**, and *acy-1* could not be detected despite using three different primer sets. We were unable to perform Western blot analysis due to lack of availability of antibodies against these *C. elegans* regulators; however, our qPCR results clearly show that **expression levels of the major learning regulator, *casy-1*, are reduced by PFF treatment.**

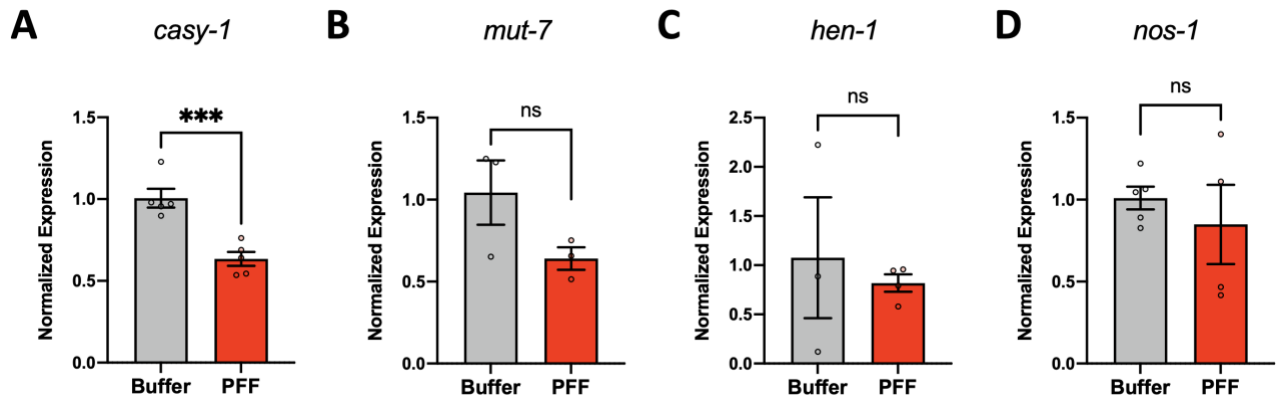


Figure 4: Expression levels of learning & memory regulators in *C. elegans* fed α-synuclein PFFs. qPCR was performed on day 4 adult *C. elegans* that had been fed PFFs or buffer (strain UM0011). Levels of learning regulators **(A-C)** and 0.5 hr memory regulators **(D)** revealed a significant reduction of *casy-1* in PFF-fed worms. Each data-point represents approximately 500 worms that were pooled for analysis.

To investigate a potential interaction of α-synuclein PFFs with CASY-1, we used a fluorescent CASY-1 reporter strain and fluorescently-labeled α-synuclein PFFs. Confocal imaging was performed to determine if the PFFs and CASY-1 are colocalized **(Figure 5)**.

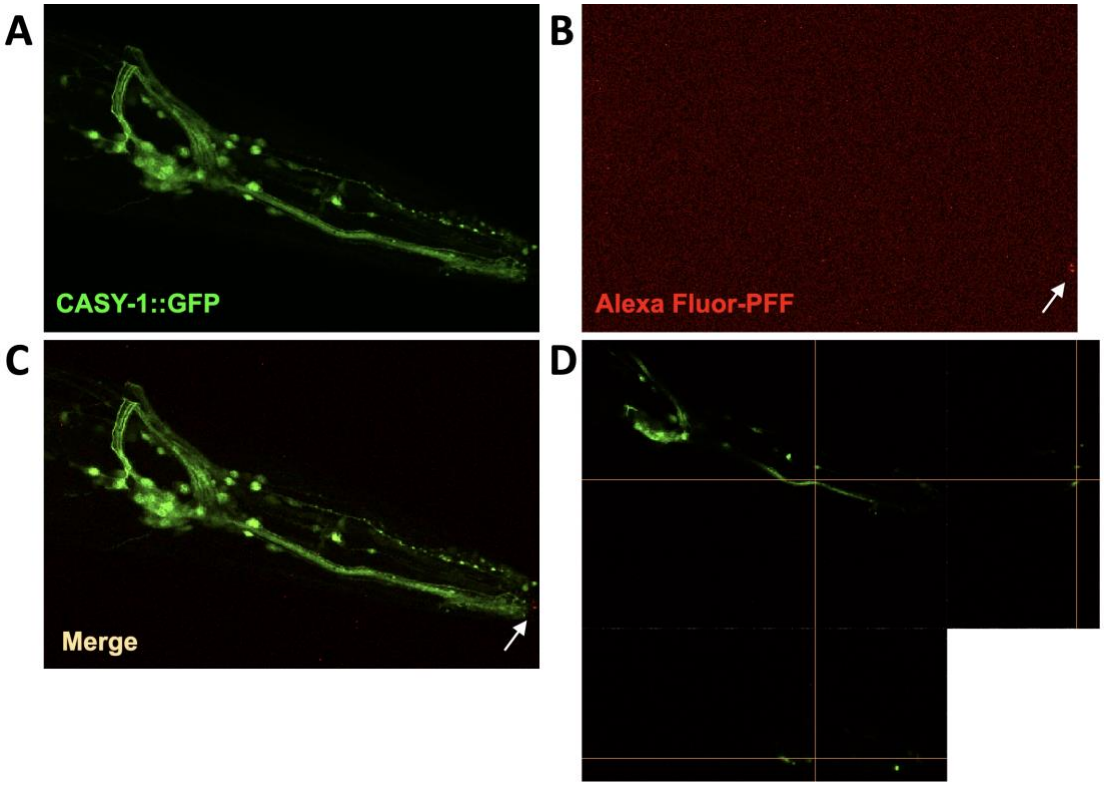
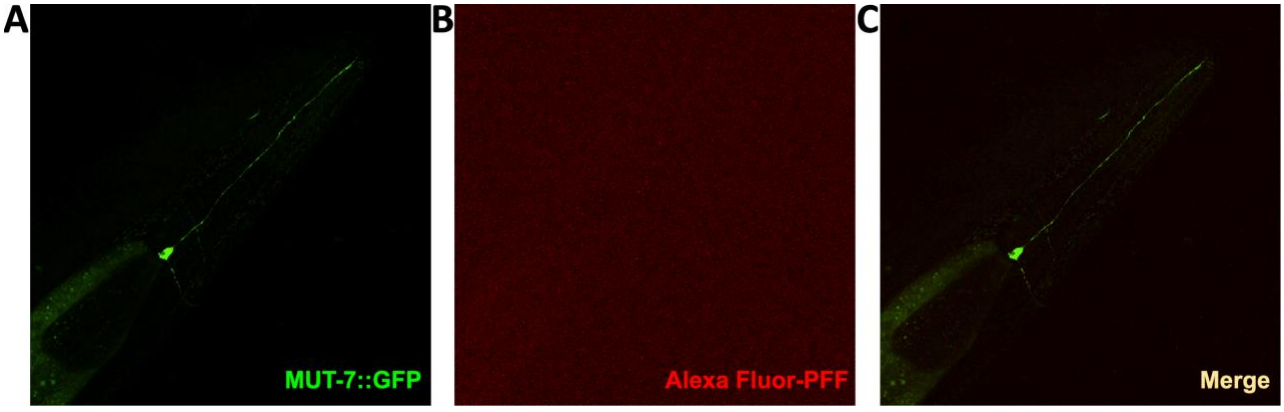


Figure 5: Testing the potential interaction of α-synuclein PFFs with the learning regulator, CASY-1. As per our published protocols[1] for imaging PFFs in *C. elegans*, worms (*cont. next page*)

(strain BC11525) were exposed to Alexa Fluor-labeled PFFs on day 1 of adulthood for a total of 24 hours, and then imaged after a 1-hr 'wash-out' period to remove excess PFFs. **(A)** GFP-labeled CASY-1 was visualized and is found in head neurons (depicted is the head region of a worm). **(B)** Labeled PFFs were visualized and residual PFFs can be seen at the mouth opening (arrow in B and C). **(C)** CASY-1 and PFF signals were merged, and each pixel was examined for possible colocalization. An example of 3D pixel mapping is shown in **(D)**. No colocalization was found.

It was found that **α-synuclein PFFs do not colocalize with CASY-1 *in vivo* (Figure 5C-D)**. Due to a lack of availability of recombinant *C. elegans* CASY-1 protein, we were unable to test for an interaction of α-synuclein with CASY-1 *in vitro*. However, in my discussions with my Co-Mentors, it was advised that, since there is no evidence of an interaction *in vivo*, that *in vitro* binding assays are not warranted. We have therefore concluded that **α-synuclein PFFs disrupt cognitive function by reducing expression levels of the learning regulator, CASY-1, but not via a direct interaction.**

During my discussions with my Co-Mentors, it was noted that α-synuclein PFFs may interact with learning & memory regulators <u>without</u> affecting their expression levels. While the approved Statement of Work did not require pursuing Subtasks 2 and 3 for any regulators whose expression levels remained unchanged, we decided to perform confocal imaging for two additional factors (MUT-7 and GLR-1) to determine if there is an interaction with α-synuclein that may contribute to the cognitive deficits caused by PFFs. Similarly to CASY-1, we used fluorescent reporter strains and fluorescently-labeled PFFs. No colocalization was found for PFFs with either MUT-7 **(Figure 6)** or GLR-1 **(Figure 7)**, suggesting that direct interactions of α-synuclein with these factors does not underlie PFF-induced cognitive impairment.



<u>Figure 6: Testing the potential interaction of α-synuclein PFFs with the learning regulator, MUT-7</u>. As per our published protocols[1] for imaging PFFs in *C. elegans*, worms (strain BC14993) were exposed to Alexa Fluor-labeled PFFs on day 1 of adulthood for a total of 24 hours, and then imaged after a 1-hr 'wash-out' period to remove excess PFFs. **(A)** GFP-labeled MUT-7 was visualized in a head neuron (depicted is the head region of a worm). **(B)** Labeled PFFs were imaged, and **(C)** MUT-7 and PFF signals were merged. As in Figure 5, each pixel was examined for possible colocalization. However, no colocalization was found.

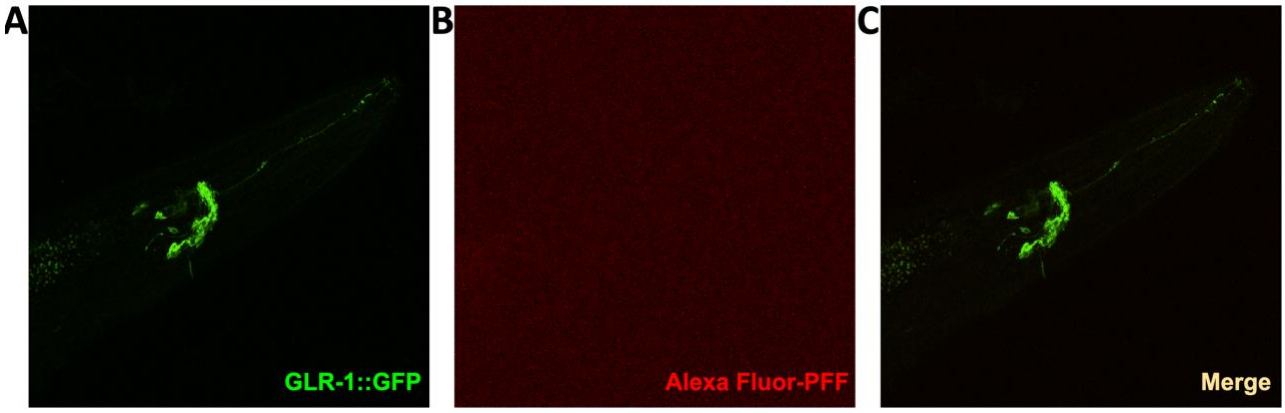


Figure 7: Testing the potential interaction of α-synuclein PFFs with the learning regulator, GLR-1. As per our published protocols[1] for imaging PFFs in *C. elegans*, worms (strain KP1148) were exposed to Alexa Fluor-labeled PFFs on day 1 of adulthood for a total of 24 hours, and then imaged after a 1-hr 'wash-out' period to remove excess PFFs. **(A)** GFP-labeled GLR-1 was visualized in head neurons (depicted is the head region of a worm). **(B)** Labeled PFFs were imaged, and **(C)** GLR-1 and PFF signals were merged. As in Figure 5, each pixel was examined for possible colocalization. However, no colocalization was found.

As a result of our investigations on learning & memory regulators, we achieved the Major Task 2 Milestones of Identifying key learning & memory factors (CASY-1) that mediate the effects of gut-derived α-synuclein on cognition, and found that there is no direct interaction between α-synuclein and these factors.

Specific Aim 2: Uncover the mechanisms by which gut-derived α-synuclein enters neurons to affect cognition

Major Task 3: Screen 17 cell surface glycoprotein receptors/regulators for those that mediate short-term memory deficits in *C. elegans* model of neurotoxic α-synuclein exposure

Subtask 1: Knock down 9 cell surface receptors individually by RNAi and test for rescue of short-term memory function

Subtask 2: Knock down 8 heparan sulfate proteoglycan (HSPG) pathway genes individually by RNAi and test for rescue of short-term memory function

Subtask 3: Present my findings from Aim 1 and preliminary data from Aim 2 at the 2023 Society for Neuroscience Conference.

For Specific Aim 2 Major Task 3, our major activity is to screen 17 specific genes for those that regulate learning & memory in the *C. elegans* disease models. In Subtask 1, the specific objective is to screen cell surface receptor genes, in Subtask 2, the specific objective is to screen proteoglycan genes, and in Subtask 3, the specific objective is to present our results from Aims 1 and 2 at a premier international scientific conference.

Significant results and key outcomes:

As per the approved SOW, this Major Task is currently underway/ in-progress at the time of submitting this Annual Report. We are actively conducting the proposed experiments and in the process of analyzing the data. We anticipate on-time completion of Major Task 3, including presenting our findings at the 2023 Society for Neuroscience Conference. This meeting will take place on November 11-15, 2023 in Washington DC, and I submitted our abstract on June 13, 2023 (please see Appendix).

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

As per the Researcher Development Plan, I have received and benefited from multiple training and professional development opportunities during the reporting period.

Training opportunities: I have received highly valuable mentorship from my two Co-Mentors, Dr. Chandramohan Wakade and Dr. Erhard Bieberich (Dr. Bieberich was approved as a replacement Co-Mentor following the passing of Dr. Robert Yu). The Mentoring Plan consists of 3 main goals:
(1) Achieve expertise in glycobiology as it pertains to neurotoxin exposure Parkinson's research.
(2) Gain translational research expertise as it pertains to treatment-related Parkinson's research.
(3) Establish a solid foundation for a career as an independent Parkinson's researcher.
Both Co-Mentors have been instrumental in helping me towards achieving the above goals. During our mentoring meetings (both one-on-one and group meetings), we have discussed progress towards completion of the 3 Aims, including data collected thus far, interpretations of the data, and planned experiments moving forward. Throughout the reporting period, as I have collected more and more data, our meetings have begun to include discussion of how the results will be presented in a manuscript and what additional experiments might be necessary for publication.

Regarding goal (1) above, Dr. Bieberich has specifically helped me to gain expertise in glycobiology as it is relevant to neurotoxin exposure Parkinson's research, especially as we have moved into Aim 2 experiments in which we are testing proteoglycan and other cell surface receptor genes. Regarding goal (2), Dr. Wakade's knowledge of translational research has been helpful to me throughout my progress on the project, as we have regularly discussed the clinical implications of the work, the potential relevance to Parkinson's patients, and how my results may translate into future clinical studies. Dr. Wakade's guidance in translational research as it pertains to treatment-related Parkinson's research will also be highly pertinent when we begin Aim 3 experiments. Regarding goal (3), both Co-Mentors have been highly supportive and helped me to find and take advantage of opportunities to establish an independent career in Parkinson's research. These opportunities have included giving a seminar, presenting my work at an international conference, publishing a Review article in a peer-reviewed journal, hosting leading Parkinson's experts in our departmental seminar series, and preparing a manuscript for publication detailing our new methodologies. (Please see the next section, "Professional opportunities" for more information.)

Professional opportunities: I have already benefited from several professional development opportunities that are helping me to establish my professional footprint in the area of neurotoxin exposure and treatment-related Parkinson's research. As a result of the knowledge I have gained in this field due to actively working on the funded project and learning from excellent mentorship,

I was able to publish a comprehensive Review article in a peer-reviewed journal, discussing the gut-to-brain hypothesis of Parkinson's disease (Chen, **Mor DE***. Gut-to-brain α-synuclein transmission in Parkinson's disease: evidence for prion-like mechanisms, *Int J Mol Sci* 2023; 24(8):7205. *corresponding author) (Please see Appendix.) Publishing this work has not only helped me to deepen my knowledge of Parkinson's disease and the neurotoxin α-synuclein, but has also helped me to establish myself as a Parkinson's expert, and an authority in the field. In support of this, my Review article has already been cited by another peer-reviewed paper only 6 weeks after initial online publication. In addition, due to the new protocols we have developed in the course of completing Aim 1, we are now preparing a manuscript detailing these new methods (please see Appendix), and we anticipate submission of this manuscript to a peer-reviewed journal by the end of summer 2023. This publication will help to guide other labs who would like to use our new *C. elegans* PD models, and will advance my professional growth, as well.

I have also had the opportunity to present my findings from the funded project to the larger scientific community, further establishing myself as an independent researcher in the Parkinson's field. This presentation was an Invited Seminar to the University of Pennsylvania Center for Neurodegenerative Disease Research, which I delivered virtually on March 30, 2023 (Talk Title: "Investigating α-synuclein transmission from the gut: new models of Parkinson's disease"). To further disseminate the results of the project thus far, and promote my independent career in Parkinson's research, I have submitted an Abstract to the Society for Neuroscience conference, which is an international meeting of neuroscientist researchers and clinicians (please see Appendix for Abstract).

With the help of my Co-Mentors, I have also hosted several leading Parkinson's disease and neurodegeneration experts in our departmental seminar series, which has fostered my highly fruitful scientific interactions with established researchers in the field. These experts were: Dr. Virginia Lee (University of Pennsylvania), Dr. Aimee Kao (University of California San Francisco), and Dr. Robert Kalb (Northwestern University). By inviting these individuals, I was able to forge new professional relationships and further establish myself as a Parkinson's disease researcher.

How were the results disseminated to communities of interest?

Thus far, the results have been disseminated to both scientific and lay (public) communities in the following ways:

Scientific communities of interest: On March 30, 2023, I gave a virtual Invited Seminar to the University of Pennsylvania Center for Neurodegenerative Disease Research, titled "Investigating α-synuclein transmission from the gut: new models of Parkinson's disease". In this presentation, I shared our Aim 1 results with the academic scientific community that specializes in neurodegenerative disease research, and had very productive discussions regarding the project methodologies and the clinical significance of our work. In addition, I have submitted an Abstract to the Society for Neuroscience conference, whose reviewers are scientific peers knowledgeable in the fields of neuroscience, and who are capable of selecting high-impact submissions for inclusion in the global meeting. This conference will take place on November 11-15, 2023 in Washington DC (please see Appendix for my Abstract).

Public communities of interest: To enhance public understanding and interest in science, my funded project was featured in two media articles that were disseminated to the University-wide community as well as the public at large: On February 28, 2023, the Augusta University JagWire published "Tiny worm plays a big role in learning whether Parkinson's really starts in the gut" which can be accessed at https://jagwire.augusta.edu/tiny-worm-plays-a-big-role-in-learning-whether-parkinsons-really-starts-in-the-gut. Following this, I received additional media coverage on March 17, 2023, when the Medical College of Georgia Medicine Magazine at Augusta University featured our funded work in "News & Views: From Gut to Brain" which can be accessed at https://magazines.augusta.edu/2023/03/17/from-gut-to-brain. I have since received multiple emails from members of the public referencing this coverage and indicating both interest and support, suggesting that our work has contributed to public understanding and engagement with scientific research.

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

During the next reporting period, our plan is the following (as per the approved Statement of Work): **Complete Specific Aim 2 Major Task 3** such that we will achieve the Milestones of Identifying glycoprotein receptors and/or regulators that mediate the effects of gut-derived α-synuclein on cognition, **Complete Specific Aim 2 Major Task 4** such that we will achieve the Milestones of Identifying whether there is a direct interaction between α-synuclein and cell surface glycoprotein receptors or regulators that mediate α-synuclein neurotoxicity, **Complete Specific Aim 3 Major Task 5** such that we will achieve the Milestones of Identifying potential therapeutics that restore cognitive function in the *C. elegans* α-synuclein neurotoxicity models, and **Complete Specific Aim 3 Major Task 6** such that we will achieve the Milestones of Determining the effects of gut-derived α-synuclein on mitochondrial function and potential therapeutics that restore normal mitochondrial activity.

I also plan to **publish our new methodologies** developed during Aim 1 (Please see Appendix), as well as **publish the results of Aims 1, 2, and 3** in peer-reviewed journals. I plan to **present our findings at the Society for Neuroscience conference** on November 11-15, 2023 in Washington DC (Please see Appendix), and I plan to **submit an Abstract for presentation at the Keystone Symposium on Neurodegenerative Diseases** that will take place on June 3-6, 2024 in Santa Fe, NM. I also plan to **host additional experts in the field** of Parkinson's disease and neurodegeneration in our departmental seminar series, including Dr. Monica Driscoll (Rutgers University) who has already accepted an invitation for an in-person visit and seminar.

I will **continue to have regular meetings (both one-on-one and group meetings) with my Co-Mentors,** who will continue to provide extremely valuable feedback and mentorship as we work towards completion of the project. **Through all of these activities, I plan to achieve the scientific goals in the Statement of Work as well as the training and professional development goals outlined in the Researcher Development Plan.** This will enable me to acquire all of the necessary skills, competence, and expertise to successfully complete the proposed research project and achieve independence in the area of neurotoxin exposure and treatment-related Parkinson's research.

4. IMPACT:

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

The project and accomplishments in the reporting period have had a positive impact on the development of the principal discipline, which is Parkinson's disease research, in the following ways: Our results from Aim 1 have shown that α-synuclein neurotoxin originating in the gut can cause learning & memory deficits in animal models, bearing significant implications for Parkinson's disease mechanisms and potential therapeutic interventions. These findings were shared with the scientific community during my Invited Seminar at the University of Pennsylvania, which inspired great interest. I anticipate that presentation of the results at the 2023 Society for Neuroscience conference will further promote research interest in this area. Publications resulting from the project are also expected to advance the field, including a Review article that I already published in which critical evidence for the gut-to-brain hypothesis of Parkinson's disease is discussed. New protocols related to our animal models are also being prepared for publication, which will facilitate other research groups adopting these models for Parkinson's disease research. Finally, the project has been featured in two media articles thus far, expanding our outreach to the general public and generating great interest in Parkinson's research and scientific understanding.

What was the impact on other disciplines?

While the project is focused on mechanisms and treatments related to Parkinson's disease, it is possible that our findings of gut-to-brain spread of disease may be relevant to other diseases originating in the gastrointestinal tract, or other synucleinopathies that share similarities with Parkinson's disease. However, the specific impact on other disciplines is not yet known.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

The ultimate goal of Parkinson's disease research, and for which the funded project is also relevant towards, is the improvement of medical treatments for this debilitating disease. Thus, the anticipated impact of the project will be to inform medical practices and therapies to improve the lives of Parkinson's disease patients among military veterans and the general American public.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report.

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

While we encountered difficulties completing some of the experiments (as described below), **in all cases very productive discussions with my Co-Mentors allowed us to strategically move forward with the best use of resources to advance the project and stay on track with the stated goals**. Specifically, the problems encountered were: long-term memory assays could not be performed due to these assays being considerably more difficult than short-term memory assays. Despite multiple attempts, consultation with the lab that created these assays (Dr. Coleen Murphy at Princeton University, with whom I did my postdoc), and extensive guidance from my Co-Mentors, we were unable to achieve reliable long-term memory in the controls (non-transgenic worms). Rather than continuing to devote time and resources towards more troubleshooting, with the support of my Co-Mentors, **we decided to focus on our exciting short-term memory data**, with which we had successfully shown neurotoxin-induced cognitive impairment and **could therefore move forward with the project and goals**.

Additional difficulties arose when measuring expression levels of learning & memory regulators, when we were unable to measure mRNA for 2/6 regulators despite using multiple primer sets, and could not perform Western blotting due to lack of availability of *C. elegans* antibodies. However, our **successful qPCR experiments showed** that 3/6 regulators have unchanged gene expression, while ***casy-1* levels are specifically reduced with PFF treatment, which was highly significant**. While we were unable to test for a direct interaction of PFFs with CASY-1 *in vitro* due to lack of availability of the *C. elegans* recombinant protein, **the more important experiment of *in vivo* interaction was successfully performed** using confocal microscopy, and revealed no interaction. It was decided with the guidance of my Co-Mentors that *in vitro* experiments with CASY-1 were not warranted, due to the lack of *in vivo* evidence to justify an interaction with PFFs. In addition, we tested 2 more regulators (MUT-7 and GLR-1) for interaction with PFFs *in vivo* using imaging, partially mitigating the lack of expression level data for one of these regulators (GLR-1).

Changes that had a significant impact on expenditures

Nothing to Report.

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

Nothing to Report.

Significant changes in use or care of human subjects

Nothing to Report.

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:

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

Journal publications.

Chen M, Mor DE. Gut-to-brain α-synuclein transmission in Parkinson's disease: evidence for prion-like mechanisms, *Int J Mol Sci* 2023; 24(8):7205; Published; Acknowledgement of federal support (Yes). Szelong K, Yerigenahally S, Mor DE. Treatment of *C. elegans* with α-synuclein pre-formed fibrils to induce Parkinson's-like disease; In preparation; Acknowledgement of federal support (Yes).

Books or other non-periodical, one-time publications.

Chen M, Szelong K, Vincent J, Ezeanii A, Wakade S, Yerigenahally S, Mor DE. Age-dependent motor and cognitive decline following neurotoxic α-synuclein exposure in new Parkinson's disease models, Abstract Submitted to Society for Neuroscience Conference on June 13, 2023; Under Review; Acknowledgement of federal support (Yes).

Other publications, conference papers and presentations.

Mor DE. Investigating α-synuclein transmission from the gut: new models of Parkinson's disease, Invited Seminar to the University of Pennsylvania Center for Neurodegenerative Disease Research, Virtual on March 30, 2023; Delivered; Acknowledgement of federal support (Yes).

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

Baker T. News & Views: From Gut to Brain. MCG Medicine Magazine at Augusta University. March 17, 2023: 10. https://magazines.augusta.edu/2023/03/17/from-gut-to-brain. Media article featuring the funded project. Baker T. Tiny worm plays a big role in learning whether Parkinson's really starts in the gut. JagWire. February 28, 2023. https://jagwire.augusta.edu/tiny-worm-plays-a-big-role-in-learning-whether-parkinsons-really-starts-in-the-gut. Media article featuring the funded project.

- **Technologies or techniques**

In the process of adapting the published *C. elegans* learning & memory protocols[2] to new protocols that can be performed with α-synuclein treatments, we have been able to prepare a manuscript detailing these new methods (please see Appendix). We anticipate submission of this manuscript to a peer-reviewed journal by the end of summer 2023. This publication will not only help to guide other labs who are using our PFF *C. elegans* models to study PD, but will continue to advance my career in the field of PD neurotoxin research.

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

Nothing to Report.

- **Other Products**

Nothing to Report. (Please see above for all reportable outcomes developed under this project.)

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Danielle Mor
Project Role:	PI
Researcher Identifier (ORCID):	0000-0003-3851-5010
Nearest person month worked:	4
Contribution to Project:	Dr. Mor has overseen the project including experimental design, data interpretation, writing of the manuscripts, and organizing meetings with the Co-Mentors.
Funding Support:	Medical College of Georgia at Augusta University Start-Up Fund
Name:	Shobha Yerigenahally
Project Role:	Research Associate
Researcher Identifier (ORCID):	0000-0002-3468-678X
Nearest person month worked:	12
Contribution to Project:	Ms. Yerigenahally is the primary person responsible for performing the experiments, analyzing data, keeping track of inventory, and record keeping.
Funding Support:	Medical College of Georgia at Augusta University Start-Up Fund

Name:	Kieran (Katherine) Szelong
Project Role:	Research Assistant
Researcher Identifier (ORCID):	
Nearest person month worked:	3
Contribution to Project:	Mr. Szelong helps to perform experiments, analyze data, and write manuscripts.
Funding Support:	Medical College of Georgia at Augusta University Start-Up Fund

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

Danielle Mor (PI, 35% effort): Nothing to Report.

Chandramohan Wakade (Co-Mentor, 0% effort): VA grant 5I01CX001815-04 has closed.

Erhard Bieberich (Co-Mentor, 0% effort): NIA grant 1R21AG078601-01 is now active.
NIA grant 1RF1AG078338-01 is now active.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A.

QUAD CHARTS: Please see Appendix.

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,*

Identifying Mechanisms and Treatments for Cognitive Decline Following Neurotoxic Alpha-Synuclein Exposure in New Parkinson's Disease Models

W81XWH-22-1-0545

PI: Danielle Mor, PhD **Org:** Augusta University Research Institute, Inc. **Award Amount:** $400,000

Study/Product Aim(s)

- Specific Aim 1: Determine the effects of gut-derived α-synuclein on learning and memory cognitive function
- Specific Aim 2: Uncover the mechanisms by which gut-derived α-synuclein enters neurons to affect cognition
- Specific Aim 3: Identify novel treatments for α-synuclein-induced cognitive dysfunction by targeting cellular metabolism

Approach

We are testing cognitive function in our new *C. elegans* Parkinson's disease models that are fed α-synuclein neurotoxin to initiate disease from the gut. We are screening highly conserved genes to determine if they facilitate α-synuclein spreading in these models, and we will test FDA-approved drugs and dietary supplements to identify those that may improve cognitive function. This work may offer new targets and potential therapies for halting neurotoxic α-synuclein spread in Parkinson's disease.

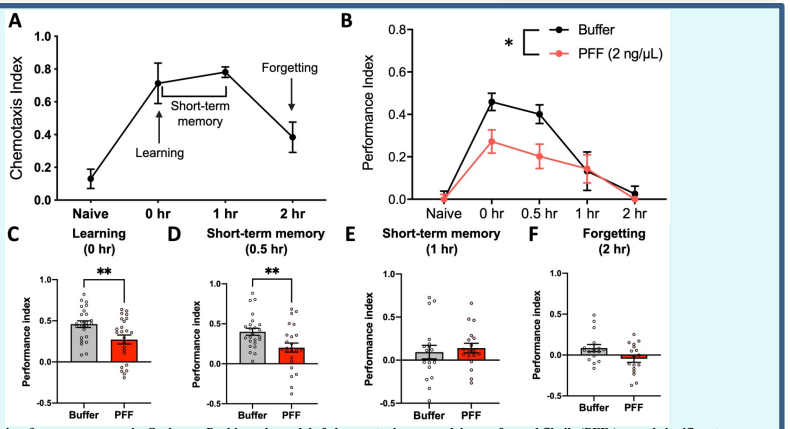


Figure 1: Learning & memory assays in *C. elegans* Parkinson's models fed neurotoxic α-synuclein pre-formed fibrils (PFFs) reveal significant cognitive deficits on day 4 of adulthood. **(A)** Example of learning & memory in non-transgenic *C. elegans*. **(B)** PFF-fed worms (strain UM0011) perform significantly worse than controls in short-term memory assays. **(C-F)** Closer examination of the different phases of learning & memory reveals specifically a learning defect **(C)** and short-term memory defect at 0.5 hours post-training for the PFF group. Each data-point represents a replicate with 30-100 worms.

Accomplishment: α-synuclein neurotoxin exposure in *C. elegans* causes significant loss of short-term learning and memory function (Figure 1), recapitulating key cognitive symptoms in Parkinson's disease.

Timeline and Cost

Activities	CY 22	23	24	
Specific Aim 1				
Specific Aim 2				
Specific Aim 3				
Estimated Budget ($K)	**$100**	**$200**	**$100**	**$000**

Updated: July 31, 2023

Goals/Milestones

CY22 Goal – Specific Aim 1

☑ Test effects of α-synuclein on cognitive function

CY23 Goals – Specific Aims 1, 2, 3

☑ Test effects of α-synuclein on cognitive function

☐ Screen genes to uncover those that mediate α-synuclein spread

☐ Screen drugs to uncover those that improve cognitive function

CY24 Goal – Specific Aim 3

☐ Screen drugs to uncover those that improve cognitive function

Comments/Challenges/Issues/Concerns

- No timeline change.
- No spending change.

Budget Expenditure to Date

Projected Expenditure: $200,000

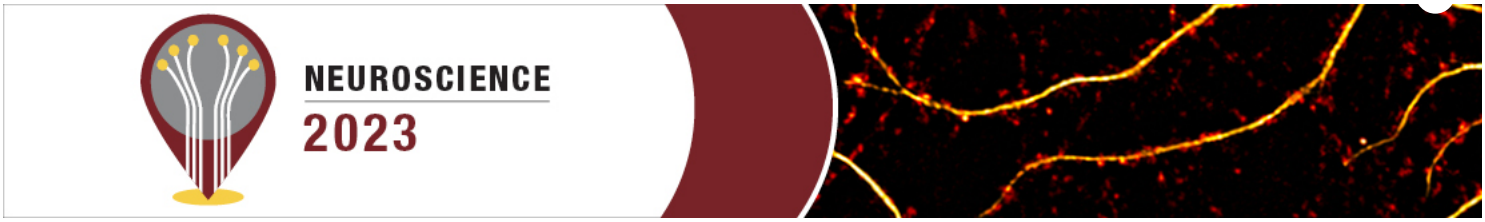
Actual Expenditure: $190,000

Bibliography:

1. Chen M, Vincent J, Ezeanii A, Wakade S, Yerigenahally S, Mor DE. Heparan sulfate proteoglycans mediate prion-like α-synuclein toxicity in Parkinson's in vivo models. Life Sci Alliance. 2022 Jul 5;5(11):e202201366. doi: 10.26508/lsa.202201366. PMID: 35790300; PMCID: PMC9259873.

2. Kauffman A, Parsons L, Stein G, Wills A, Kaletsky R, Murphy C. C. elegans positive butanone learning, short-term, and long-term associative memory assays. J Vis Exp. 2011 Mar 11;(49):2490. doi: 10.3791/2490. PMID: 21445035; PMCID: PMC3197297.

3. Stein GM, Murphy CT. C. elegans positive olfactory associative memory is a molecularly conserved behavioral paradigm. Neurobiol Learn Mem. 2014 Nov;115:86-94. doi: 10.1016/j.nlm.2014.07.011. Epub 2014 Aug 7. PMID: 25108196; PMCID: PMC4250358.



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Activity: Scientific Abstract
Current Date/Time: 6/13/2023 2:53:07 PM

Age-dependent motor and cognitive decline following neurotoxic α-synuclein exposure in new Parkinson's disease models

AUTHOR BLOCK: M. CHEN[1], K. SZELONG[1], J. VINCENT[1], A. EZEANII[1], S. WAKADE[2], S. YERIGENAHALLY[1], **D. E. MOR**[1];
[1]Augusta Univ., Augusta, GA; [2]Georgia Inst. of Technol., Atlanta, GA

Abstract:
Parkinson's disease (PD) is a debilitating neurodegenerative disorder characterized by both motor and cognitive symptoms and the formation of pathological inclusions containing aggregated α-synuclein protein. The gut-to-brain hypothesis of PD suggests that α-synuclein aggregation may originate in the gastrointestinal tract, potentially due to environmental toxin exposures, and thereafter spread to the central nervous system in a prion-like fashion. While rodent models have demonstrated that gut-to-brain α-synuclein spreading can occur, the precise mechanisms of α-synuclein transmission and resulting neurotoxicity remain largely unknown. Animal models that are amenable to rapid, high-throughput investigation are needed in order to facilitate the discovery of disease mechanisms and potential therapeutic targets. To this end, we have developed new 'gut-to-brain' PD models using the small model organism, *C. elegans*. *C. elegans* have a well-defined nervous system with highly conserved neurotransmitter signaling, a diverse set of motor and cognitive behaviors, and high genetic tractability. To initiate α-synuclein spreading from the gut, worms are exposed to an exogenous source of wild-type human α-synuclein pre-formed fibrils (PFFs). We have shown that PFFs are ingested, spread to body tissues, and induce age-dependent motor dysfunction that is dependent on host expression of human α-synuclein in neurons or muscle, consistent with prion-like mechanisms. PFF feeding also promotes dopamine neuron degeneration and the aggregation of host α-synuclein, recapitulating key features of PD. Furthermore, PFF exposure causes age-dependent loss of learning and memory function, offering new models of cognitive decline in PD. To identify mechanisms by which gut-derived α-synuclein may enter neurons and cause toxicity, we conducted a targeted RNAi screen and showed, for the first time *in vivo*, that heparan sulfate proteoglycans regulate disease phenotypes caused by α-synuclein PFFs. The results of this study offer new tools and potential targets for PD therapeutics aimed at halting the neurotoxic spread of α-synuclein from the gut to the brain.

Author Disclosure Information:

M. Chen: None. **K. Szelong:** None. **J. Vincent:** None. **A. Ezeanii:** None. **S. Wakade:** None. **S. Yerigenahally:** None. **D.E. Mor:** None.
Presentation Preference (Complete): Nanosymposium Preferred
Linking Group Selection (Complete): None selected
Theme and Topic (Complete): C.03.f. Alpha-synuclein - Mechanisms and transmission
Linking Group and Nano Info (Complete):
Keyword (Complete): ALPHA-SYNUCLEIN ; PARKINSON'S DISEASE ; C. ELEGANS
Support (Complete):
Support: Yes
Grant/Other Support: : Department of Defense PD210045 W81XWH-22-1-0545
Grant/Other Support: : Medical College of Georgia at Augusta University Start-Up Fund

Special Requests (Complete):
How do you plan to participate in Neuroscience 2023?: In-Person Only
Religious Conflict?: No Religious Conflict
Additional Conflict?: No
Is the presenting author of this abstract a high school or undergraduate student?: None
Select: No preference

Status: Complete

International Journal of
Molecular Sciences

MDPI

Review

Gut-to-Brain α-Synuclein Transmission in Parkinson's Disease: Evidence for Prion-like Mechanisms

Merry Chen and Danielle E. Mor *

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* Correspondence: dmor@augusta.edu

Abstract: Parkinson's disease (PD) is a multifactorial disorder involving both motor and non-motor symptoms caused by the progressive death of distinct neuronal populations, including dopaminergic neurons in the substantia nigra. The deposition of aggregated α-synuclein protein into Lewy body inclusions is a hallmark of the disorder, and α-synuclein pathology has been found in the enteric nervous system (ENS) of PD patients up to two decades prior to diagnosis. In combination with the high occurrence of gastrointestinal dysfunction in early stages of PD, current evidence strongly suggests that some forms of PD may originate in the gut. In this review, we discuss human studies that support ENS Lewy pathology as a characteristic feature of PD, and present evidence from humans and animal model systems that α-synuclein aggregation may follow a prion-like spreading cascade from enteric neurons, through the vagal nerve, and into the brain. Given the accessibility of the human gut to pharmacologic and dietary interventions, therapeutic strategies aimed at reducing pathological α-synuclein in the gastrointestinal tract hold significant promise for PD treatment.

Keywords: alpha-synuclein; Parkinson's disease; enteric nervous system; prion-like

Citation: Chen, M.; Mor, D.E. Gut-to-Brain α-Synuclein Transmission in Parkinson's Disease: Evidence for Prion-like Mechanisms. *Int. J. Mol. Sci.* **2023**, *24*, 7205. https://doi.org/10.3390/ijms24087205

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Accepted: 11 April 2023
Published: 13 April 2023

1. Introduction

With a continually increasing disease burden of nearly 10 million patients worldwide [1], Parkinson's disease (PD) is a devastating neurodegenerative disorder that is characterized by the loss of multiple neuronal populations and the aggregation of α-synuclein protein into intracellular inclusions known as Lewy bodies (LBs) [2,3]. The progressive death of dopaminergic neurons in the substantia nigra leads to a depletion of dopamine signaling in the striatum that manifests as classic Parkinsonian symptoms, such as bradykinesia, rigidity, and resting tremors which worsen over time [4]. In addition to impaired movement, PD patients also often experience non-motor symptoms, including depression, hyposmia, difficulty sleeping [5], dementia [6], and gastrointestinal issues [7]. Autonomic dysfunction can appear decades before the onset of motor signs [5,7], highlighting the complexity of the disorder and offering potential early points of intervention. While there is currently no cure for PD, administration of medications such as the dopamine precursor, levodopa, or surgical therapies such as deep brain stimulation can provide symptomatic relief, along with available treatments for non-motor indications.

The high occurrence of gastrointestinal dysfunction in the early stages of PD [7], coupled with increased recognition of PD gut microbiome dysbiosis [8] and repeated observations of α-synuclein pathology in the enteric nervous system (ENS) of PD patients [9–23], have collectively inspired great interest in the possibility that the disorder may originate in the gut. The gut-brain axis is a complex bidirectional signaling network by which the brain communicates with the gastrointestinal tract via the ENS that relays both sensory and motor information, bacteria-derived neuroactive molecules, and

microbiome-induced cytokine release [8]. Disruption of the gut-brain axis can lead to a range of disorders from irritable bowel syndrome to functional gastrointestinal disorders, as well as potentially mood disorders and chronic pain [24]. In PD, patients experience a multitude of clinical symptoms that span the entirety of the gastrointestinal tract, including drooling, swallowing difficulties, delayed gastric emptying, small intestinal bacterial overgrowth, and constipation [7]. In addition, PD gut microbiota display an enrichment of species in the *Christensenella* [25], *Akkermansia* [26], and *Lactobacillus* [25,27] genera; depletion of species in *Bacteroides* [25,27], *Clostridium* [26,27], and *Faecalibacterium* [28,29] genera; and decreased levels of short-chain fatty acids [28]. Given that PD is a neurological disorder, these findings are consistent with gut-brain axis disturbances that may play a role in PD pathogenesis.

α-Synuclein aggregation is a hallmark of PD, yet the relationship of protein aggregation and neurodegeneration is still unclear despite extensive research efforts. Rare mutations in the α-synuclein gene, *SNCA*, cause early-onset forms of familial PD [30–35], and in sporadic PD (which accounts for at least 85% of cases), wild-type α-synuclein protein accumulates into LB inclusions and Lewy neurite (LN) axonal deposits [3,36]. In 2003, Braak et al. [37] hypothesized that PD could be staged by a topographical progression of α-synuclein lesions, the first of which appear in the dorsal motor nucleus (DMN) of the vagal nerve in the brainstem of pre-Parkinsonian patients. α-Synuclein pathology then spreads until it reaches the substantia nigra, coinciding with motor symptoms, and ultimately invades the neocortex, when patients may present with cognitive decline [36,37]. This progressive buildup of pathology across interconnected brain regions is consistent with prion-like transmission of α-synuclein from cell–cell, for which overwhelming evidence now exists in animal and cell culture model systems [38–50]. Furthermore, the early involvement of the DMN of the vagus, which serves as a major connection between the brain and the periphery, suggests that α-synuclein aggregates may first form in the ENS and gain access to the central nervous system (CNS) via the vagal nerve [14]. In this review, we will critically evaluate the evidence that ENS pathology may play a causative role in PD, with a focus on human studies and mammalian animal models of prion-like α-synuclein transmission. We will also bring to light the current gaps in knowledge and present new tools, including gut-to-brain *C. elegans* models, for advancing the scientific understanding of PD.

2. The Human Enteric Nervous System

The ENS is an intricate network of neuronal cell bodies and fibers that perform a wide array of digestive functions, including moving food through the gastrointestinal tract, facilitating nutrient uptake, regulating local blood flow, and supporting the immune system [51]. While the ENS is able to function independently of the CNS, bidirectional communication between the ENS and CNS serves to relay important information that ultimately affects organismal behavior and gastrointestinal functioning. The gut-brain axis involves the delivery of sensory information to the brain via spinal and vagal afferent pathways, and efferent motor signals to the gut by way of sympathetic and parasympathetic divisions of the autonomic nervous system [52].

Parasympathetic innervation via the vagal nerve originates in preganglionic neurons of the DMN, which synapse onto postganglionic neurons of the ENS. The nucleus ambiguus in the brainstem also supplies vagal motor efferents specifically to the pharynx and esophagus [53]. Vagal innervation of the ENS is densest in the upper gastrointestinal tract at the level of the esophagus and stomach and decreases more distally. With little to no vagal input to the distal colon and rectum, these regions are primarily regulated by the sacral parasympathetic nucleus of the spinal cord. The activity of the DMN and nucleus ambiguus can be modulated by sensory information that is relayed from the ENS

through vagal afferent pathways to the nucleus of the solitary tract in the brainstem [53]. While the influence of parasympathetic pathways can result in both enhancement and suppression of gut motility, sympathetic innervation of the digestive tract mainly acts to inhibit motility as a pro-survival reflex that is mediated by prevertebral sympathetic ganglia [54].

Within the ENS, two main neuronal networks perform the complex integration of all local (intrinsic) neuronal activity, input from extrinsic sympathetic and parasympathetic neurons, as well as cues from the gastrointestinal environment. These networks, known as the myenteric (or Auerbach's) plexus, and the submucosal (or Meissner's) plexus, use the integrated input to determine their own sensory, motor, and secretory output. The ENS is located in the gastrointestinal wall, which is comprised of four main layers: the mucosa, submucosa, muscular layer, and adventitia or serosa (Figure 1). The layer closest to the lumen of the gut, the mucosa, can be further divided into the epithelium, which forms the lining of the mucosa; the lamina propria, which is made up of connective tissue; and the muscularis mucosae, a layer of smooth muscle. Adjacent to the mucosa, the submucosa contains connective tissue, lymphatic and blood vessels, and Meissner's plexus, which exists predominantly in the walls of the small intestine and the colon. The next layer, the muscular layer, consists of circular and longitudinal smooth muscle sublayers, with Auerbach's plexus situated in between the sublayers and present along the entire length of the gastrointestinal tract. The final layer of the gastrointestinal wall is mainly connective tissue, either adventitia or serosa, depending on whether there is attachment to the surrounding organs.

Within Auerbach's and Meissner's plexuses, there are a multitude of neuronal subtypes (Figure 1). In Auerbach's plexus of the muscular layer, local motor circuits function to control gastrointestinal tract motility. These circuits are comprised of excitatory and inhibitory motor neurons that cause contraction and relaxation, respectively, of both the circular and longitudinal muscles. The excitatory motor neurons primarily use acetylcholine as their neurotransmitter, but also use tachykinins and other signaling molecules, while the inhibitory motor neurons use vasoactive intestinal polypeptide (VIP) and nitric oxide in addition to other neurotransmitters [55]. The excitatory and inhibitory motor neurons receive local input from myenteric interneurons and sensory information from intrinsic primary afferent neurons (IPANs). They are also subject to extrinsic modulation by cholinergic parasympathetic efferents arising from the preganglionic neurons of the DMN of the vagus. These vagal fibers are organized into parallel pathways that innervate either excitatory postganglionic motor neurons or inhibitory postganglionic motor neurons in the ENS [53].

In addition to this circuitry, VIP- or acetylcholine-producing secretomotor and vasodilator neurons can be found primarily in Meissner's plexus, with innervation from IPANs, interneurons, and extrinsic signals from vagal efferent pathways. Unlike the parallel excitatory and inhibitory pathways from the DMN of the vagus to the motor circuits of the ENS, vagal innervation of secretory ENS neurons is primarily excitatory [53]. Secretomotor neurons control gastrointestinal secretions, while vasodilator neurons synapse onto local arterioles and regulate blood flow. Other ENS neuron types include intestinofugal neurons that synapse onto sympathetic ganglia, and motor neurons that innervate the muscularis mucosae [55].

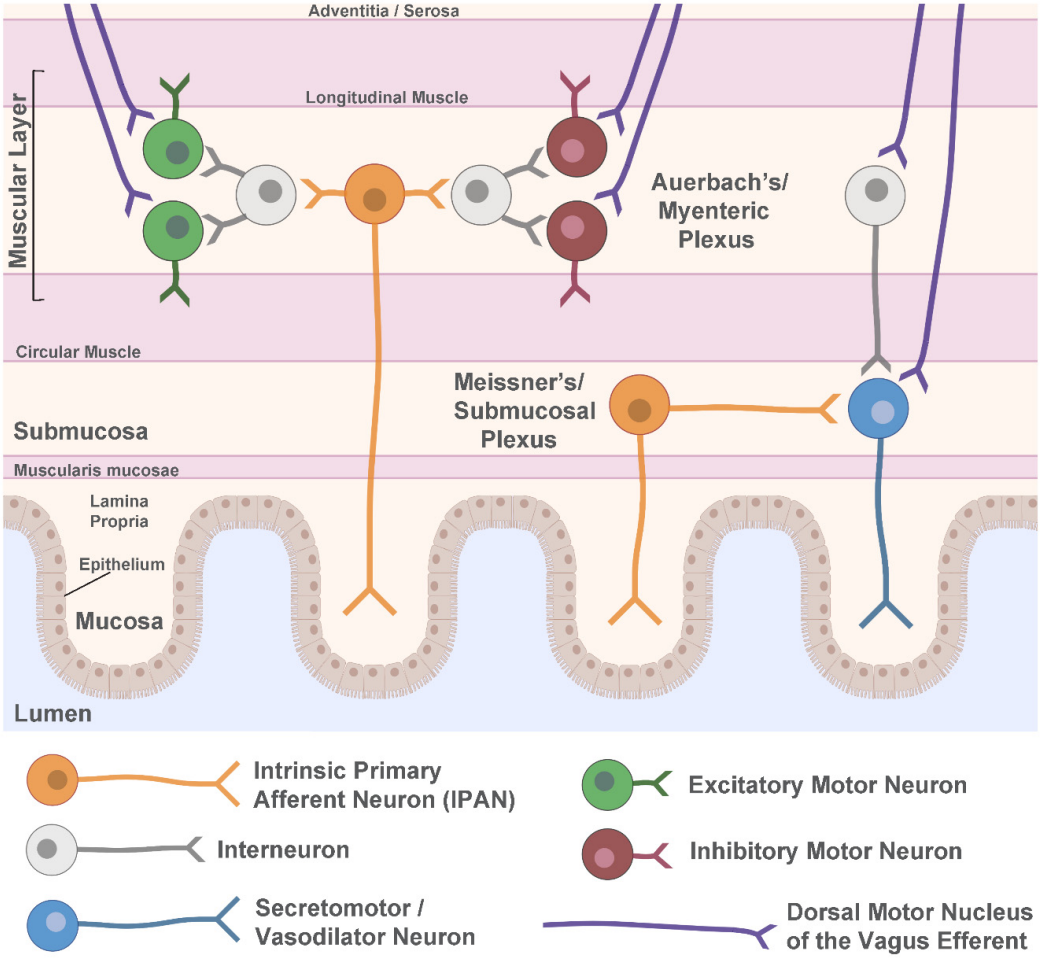

Figure 1. Schematic of the enteric nervous system. Shown are the major layers of the gastrointestinal wall with a simplified representation of neuronal circuitry. The myenteric (Auerbach's) plexus contains motor circuits that control contraction and relaxation of the muscle layer whereas secretomotor and vasodilator neurons are primarily in the submucosal (Meissner's) plexus and control local blood flow and secretion. Both plexuses receive extrinsic parasympathetic innervation from the dorsal motor nucleus of the vagus to help regulate gut motility. Sympathetic innervation and vagal sensory afferents are not shown. Figure created using Biorender.com.

3. Lewy Pathology in the Enteric Nervous System in PD

The earliest report of LB pathology in the ENS of PD patients was published in 1984 by Qualman et al. [9], who documented LBs in Auerbach's plexus of the colon from one PD patient and the esophagus from another PD patient. The esophageal LBs were associated with ganglion cell degeneration. In 1987, Kupsky et al. [10] found LBs in the ganglion cells of both Auerbach's and Meissner's plexuses of the colon and rectum in a PD patient with megacolon. Following this work, a series of studies by Wakabayashi and colleagues found that LB pathology in PD is widely distributed throughout the ENS from the upper esophagus to the rectum in both Auerbach's and Meissner's plexuses [11–13]. The greatest LB burden was found in the Auerbach's plexus of the lower esophagus [11–13], and LBs were primarily found in VIP-producing neurons although there was also rare colocalization of pathology with tyrosine hydroxylase-positive processes [12], which may correspond to noradrenergic sympathetic fibers. No loss of enteric neurons was noted [11,12].

A few years later in 1997, it was discovered that a principal component of LBs is the presynaptic protein, α-synuclein [3], and immunohistochemistry against α-synuclein or its modified forms quickly became the gold-standard for the detection of Lewy pathology. Braak and colleagues [37] used α-synuclein staining to define six stages of PD based on

the stereotypical distribution of Lewy pathology in PD patient brains. This staging scheme postulated that, in early stages of disease, focal pathology is present in a small number of circumscribed brain regions, but as the disease progresses, pathology becomes more widespread, with the recruitment of additional areas in each advancing stage. In the first stage, Lewy pathology is present in the DMN of the vagal nerve, anterior olfactory nucleus, and olfactory bulb. In Stage 2, areas such as the raphe nuclei and locus coeruleus become affected, and in Stage 3, the amygdala, basal forebrain, and substantia nigra pars compacta begin to show α-synuclein lesions. The cerebral cortex becomes involved in Stage 4, with the mesocortex affected first and the neocortex showing pathology in Stages 5 and 6 [37].

Since the brain regions affected in the Braak PD staging scheme are synaptically interconnected, and one of the earliest afflicted regions is the DMN of the vagus connecting the brain to the ENS, Braak and colleagues further investigated α-synuclein in the ENS of autopsy cases staged for Lewy pathology in the CNS [14]. In total, ten cases were examined, three of whom had been diagnosed with sporadic PD and harbored Stage 4 or 5 brain pathology, two of whom were considered incidental or presymptomatic cases having Stage 2 or 3 PD pathology, and the rest having no evidence of Lewy pathology. α-Synuclein inclusions were found in the gastric wall and the DMN of all confirmed PD and incidental cases but none of the controls, and lesions were observed in both Auerbach's and Meissner's plexuses among the PD cases [14]. The presence of Lewy pathology in the ENS of incidental cases, which harbored early-stage PD brain pathology, is consistent with ENS involvement early in PD. Yet, the small number of cases examined precludes the ability to make conclusions regarding sequential involvement of the ENS and CNS.

Following these foundational studies, several investigations were undertaken to determine the extent to which ENS pathology is a characteristic of PD. Beach et al. [16] examined phosphorylation of α-synuclein (a disease-associated modification) in the peripheral nervous system of patients with LB disorders and controls. A total of 11/17 PD patients (65%) had α-synuclein pathology in the gastrointestinal tract compared with 0/23 controls. Consistent with earlier findings of LB distribution in PD [11–13], phospho-α-synuclein staining was more concentrated in the esophagus than in lower gastrointestinal regions, and Auerbach's plexus appeared to be affected to a greater extent than Meissner's plexus [16]. In a follow-up study [18], Lewy pathology was identified in the ENS of 13/13 PD patients and none of the controls, although the number of overlapping patients between the two studies is unclear [16,18]. LBs were not found in nitric oxide or VIP neurons, except for a single VIPergic neuron in Auerbach's plexus that contained a LB [18]. There was low frequency (~3%) colocalization with tyrosine hydroxylase-positive fibers, but otherwise the cell types harboring LBs were not identified. In addition, neurodegeneration in Auerbach's plexus was carefully examined along the length of the gastrointestinal tract in PD and controls, and the authors found no neuronal loss in PD [18]. This is in contrast to a different group which found that in 15/20 PD patients, but not in controls, intestinal ganglion cells showed atrophy and/or pycnotic nuclei, and all patients with ENS degeneration also had intestinal α-synuclein deposits [22]. The question of whether Lewy pathology in the ENS is associated with local neurodegeneration thus remains unsettled.

To date, the largest postmortem study of the ENS in PD patients and controls was conducted using the Brain Bank for Aging Research (BBAR) in Japan [23]. The lower esophagus was examined from 46 PD patients, which included 38 who were diagnosed with PD dementia and the related disorder, Dementia with Lewy Bodies. A total of 340 controls who did not have Parkinsonism, dementia, or evidence of degeneration in the locus coeruleus or substantia nigra were used for comparison. Remarkably, LBs and LNs were observed in 41/46 PD subjects (89%) compared with 0% of the controls. Since the upper alimentary canal was previously found to have greater LB burden in PD than lower regions [11–13,16], the BBAR study of the esophagus can be regarded as a highly relevant sampling of tissue. Across all postmortem studies of the ENS reviewed here, PD patients had more Lewy pathology in the esophagus, stomach, small intestine, large intestine, and rectum, than controls (Figure 2A), and the total number of PD patients harboring LBs in any

segment of the gastrointestinal tract (165/212; 78%) far exceeds that in controls (17/618; 3%) (Figure 2B). These findings, therefore, support the notion that α-synuclein aggregation in the ENS is a characteristic feature of PD.

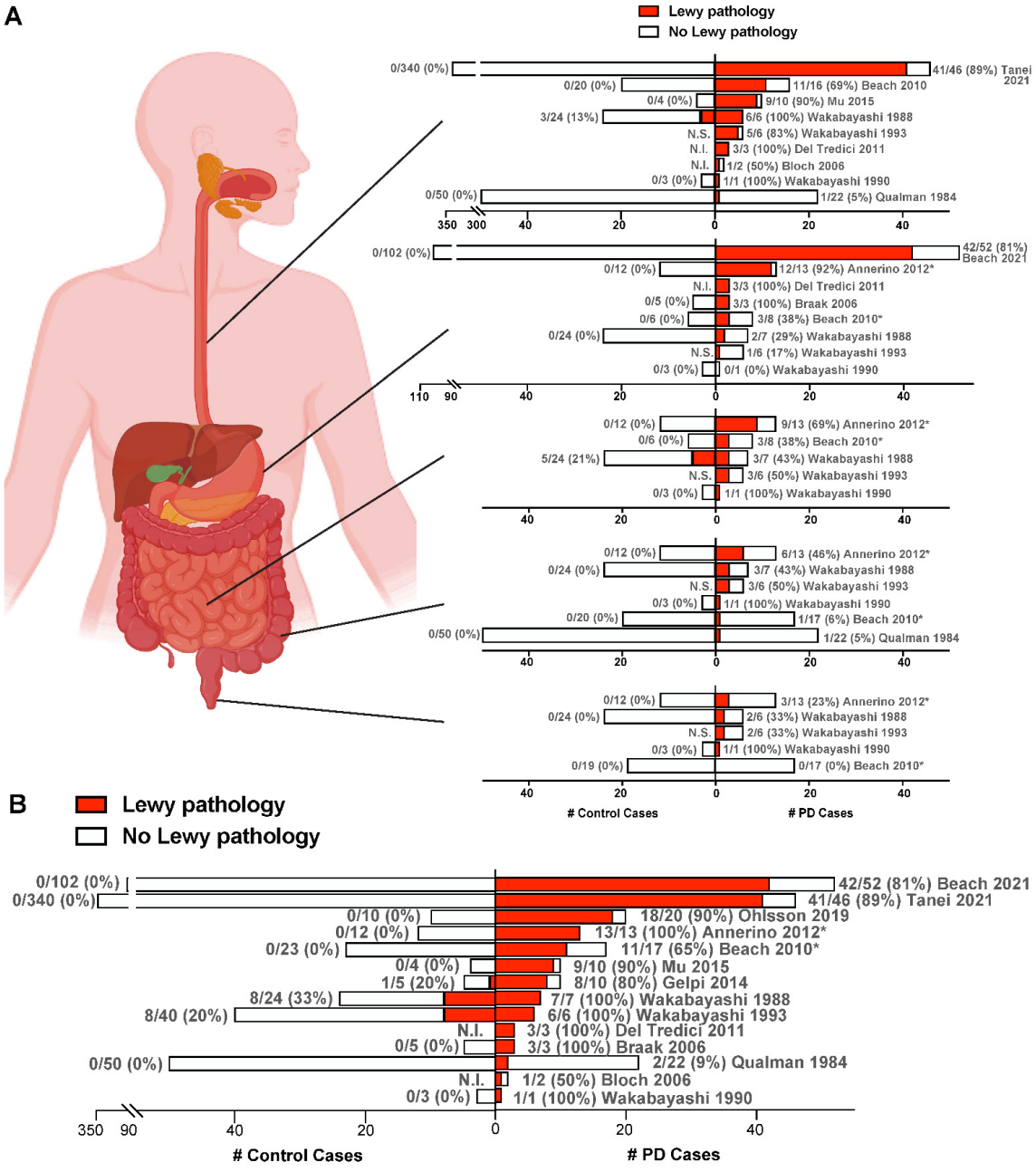

Figure 2. ENS Lewy pathology detected in postmortem studies of confirmed PD cases. (**A**,**B**) Each row represents one study, with the citation indicated. PD cases are to the right of the y-axis, while control cases are to the left of the y-axis. For each patient group, the number of cases with Lewy pathology (red) out of the total number of cases examined in that group is given next to the corresponding bar, with % positive for Lewy pathology given in parentheses. Only cases unique to each study are included. In (**A**), graphs (from top to bottom) represent studies of the esophagus, stomach, small intestine, large intestine, and rectum. In (**B**), for each study, the total number of PD or control cases with Lewy pathology detected in any gastrointestinal segment is shown. * Indicates an unknown number of subjects from these studies are overlapping. N.I., Not included in the study. N.S., Not shown in the study. Figure created using Biorender.com and GraphPad Prism 9. Refs. [9,11–20,22,23,56].

4. Does α-Synuclein Aggregation Begin in the Gut and Spread to the Brain?

Despite ample evidence of ENS pathology in PD and the hypothesis that it may precede affliction of the CNS [14], it remains unknown if α-synuclein aggregation follows a prion-like spreading cascade from enteric neurons, through the vagal nerve, and into the brain. α-Synuclein exhibits many prion-like properties, including the ability of aggregated conformations to self-replicate by inducing, or 'seeding', the aggregation of physiological α-synuclein [57,58]. In this way, the aggregate load within a cell can become amplified. In addition, similar to prions, α-synuclein can escape from one cell and infect a neighboring cell, as has been shown in culture systems and rodent transplantation studies in which host α-synuclein was detected in grafted cells [47,49]. Strikingly, postmortem studies of PD patients who received fetal mesencephalic grafts into the striatum showed that 11–16 years following transplantation, grafted neurons contained LB-like α-synuclein inclusions [59,60]. Given the young age of the grafted cells, it is unlikely that pathology developed independently of exposure to α-synuclein and/or other factors from the diseased host tissue, thereby suggesting that cell–cell transmission of α-synuclein in humans is possible.

4.1. Evidence from Incidental LB Disease and Prodromal PD

Incidental LB disease (ILBD) is thought to be a precursor to the development of PD, with subclinical LB pathology in the DMN of the vagus or other brain regions consistent with early Braak PD stages. If ILBD is in fact an incipient form of PD, then evaluation of the ENS for α-synuclein inclusions may shed light on whether protein aggregation in PD is of central or peripheral origin. Indeed, this was the motivation for Bloch et al. [15], who examined 17 cases of ILBD postmortem and found 14 of them (82%) had α-synuclein pathology in the ENS of the esophagus. Lesions were found mainly in Auerbach's plexus but also in Meissner's plexus [15]. In a larger study, esophageal Lewy pathology was found in 36/103 (35%) of preclinical or prodromal LB disease subjects on autopsy compared with 0/340 controls [23]. Several additional postmortem reports have documented Lewy pathology in the ENS of ILBD cases, albeit with very small sample sizes [9,13,14,16–18], including one study that noted LBs or LNs in the stomach, small intestine, large intestine, and rectum from at least one ILBD case for each gastrointestinal segment [18]. In general, ILBD cases have tended to have less severe LB pathology than diagnosed PD cases [16,18].

While these findings collectively suggest that α-synuclein aggregates may be present in the ENS at early PD stages, consistent with a peripheral initiation of pathology, it is not possible to confirm a specific sequential progression of α-synuclein disease from postmortem (endpoint-only) studies. Stokholm et al. [61], therefore, examined tissue blocks collected during routine gastrointestinal biopsies and surgical resections from patients who were later diagnosed with PD (on average, 7 years from diagnosis). Phosphorylated α-synuclein was observed in 22/39 (56%) of these prodromal PD patients, which was significantly higher than controls (23/90, 26%). Strikingly, α-synuclein pathology was detected in neuronal structures of the gastrointestinal tract up to 20 years prior to PD diagnosis, suggesting that ENS involvement might precede that in the CNS by decades [61]. Importantly, however, concurrent analysis of ENS and brain pathology is not possible in living patients, and, therefore, it is still not known if inclusions exist in the human ENS prior to their appearance in the CNS.

4.2. Evidence from Human Vagotomy Studies

Besides the presence of Lewy pathology in the ENS, another key aspect of the gut-to-brain hypothesis of PD is the transmission of α-synuclein through the vagal nerve. The higher density of ENS pathology in the esophagus and stomach compared to lower regions

in PD [11–13,16] is consistent with the high concentration of vagal inputs to these areas. PD patient vagotomy studies may, therefore, shed light on the dependence of the disease on intact vagal innervation of the gut. A former treatment for peptic ulcer, vagotomy, is the severing of the vagal nerve either fully (as in truncal vagotomy), solely to the stomach (selective vagotomy), or most selectively to the fundus and body of the stomach only (superselective vagotomy). In 2015, Svensson et al. [62] analyzed the risk of developing PD in over 11,000 patients catalogued in the Danish National Patient Registry as having undergone a vagotomy procedure. Truncal and selective vagotomy patients were grouped as one cohort (n = 5339), but despite this potential limitation, this group was found to have reduced risk of developing PD compared with over 60,000 controls. Notably, the effect was strongest for subjects with over 20 years of follow-up from vagotomy [62], consistent with a long latency of prodromal disease. Superselective vagotomy, which spares some connections between the stomach and the brain, as well as intestinal vagal innervation, was not associated with protection from PD [62], potentially due to the remaining vagal routes available for α-synuclein transmission.

Although these findings were challenged by a group that re-analyzed data from the same source [63], the re-examination had significant flaws. Importantly, truncal and selective vagotomies were regarded as two separate groups despite a known lack of reliability for these classifications in the Danish registry [62]. In addition, there was no minimum cut-off time from vagotomy to PD diagnosis, thereby potentially including prodromal PD patients who received vagotomy too late for their disease to be modified [64]. Moreover, a different group analyzing over 9,000 vagotomy patients in a Swedish cohort also found that truncal vagotomy reduced the risk of PD [65]. Thus, while only a few studies have examined the potential relationship of vagal denervation with PD, the findings generally support a protective effect, potentially by reducing the ability of α-synuclein to invade the CNS.

4.3. Prion-like Transmission of α-Synuclein in Rodents and Monkeys

There is now extensive evidence from animal models supporting the theory of prion-like transmission of α-synuclein in PD and other synucleinopathies. In mice and rats, intracerebral inoculation of recombinant α-synuclein pre-formed fibrils (PFFs), brain tissue from symptomatic α-synuclein transgenic mice, or brain tissue from human synucleinopathy patients, results in widespread deposition of LB-like inclusions that are often associated with neurodegeneration and motor dysfunction [38,40–42,45–47,50]. Moreover, it appears that, regardless of the site of injection, aggregation propagates along synaptic connections and requires the presence of endogenous α-synuclein, similar to prions [38,40,42,45,46,50]. In macaque monkeys, injection of PD brain tissue containing insoluble LBs into the substantia nigra or striatum caused a loss of striatal terminals followed by dopamine neuron death and diffuse α-synuclein deposits in the remaining nigral cells [40]. Other studies have also shown that α-synuclein pathology can spread to the CNS following intravenous [38], intramuscular [39], or intraperitoneal [66] injection of recombinant α-synuclein aggregates into rodents.

Importantly, the ability of α-synuclein to propagate from the gut to the brain has also been demonstrated in rodent models. In one study, Holmqvist et al. [67] injected PD substantia nigra lysate or recombinant human α-synuclein fibrils into the intestinal wall of adult rats. In both cases, at 12 h post-injection, human α-synuclein was detected in the intestinal wall but not in the vagal nerve. By 48 and 72 h post-injection, however, human α-synuclein immunoreactivity could be seen in the vagal nerve of animals injected with PD lysate or recombinant fibrils, but not in controls that were injected with bovine serum albumin. Human α-synuclein was also detected in the DMN of the vagus following intestinal injection of either PD brain lysate, or monomeric, oligomeric, or fibrillar forms

of recombinant α-synuclein [67]. These data strongly support the ability of α-synuclein of gastrointestinal origin to spread via the vagal nerve to the DMN in the brainstem. Similarly compelling evidence for this path of gut-to-brain α-synuclein spread was reported by Kim et al. [68], who injected mouse α-synuclein PFFs into the pylorus region of the stomach and duodenum of the small intestine in wild-type mice. Following gastrointestinal PFF injection, phosphorylated α-synuclein in the CNS was first detected in the DMN of the vagus and then in the locus coeruleus, amygdala, substantia nigra, and eventually the prefrontal cortex, closely mirroring the Braak staging scheme for PD. The accumulation of α-synuclein pathology was also associated with a progressive loss of dopaminergic neurons, motor dysfunction, and cognitive symptoms. Remarkably, animals that underwent truncal vagotomy or were lacking endogenous α-synuclein were entirely protected from the spread of α-synuclein pathology and any of its associated toxicities [68]. These findings further support the notion that gut-derived α-synuclein is capable of propagating through the vagal nerve in a prion-like manner to induce CNS disease.

Additional studies provide further evidence in favor of this hypothesis. Challis et al. [69] found that PFF inoculation into the duodenum of aged mice causes gastrointestinal dysfunction and promotes α-synuclein pathology in the brainstem, coupled with motor decline and decreased striatal dopamine. In two reports from Uemura et al. [70,71], injection of PFFs into the gastric wall of either wild-type [70] or α-synuclein transgenic mice expressing the A53T familial PD mutation [71] resulted in phosphorylated α-synuclein lesions in the DMN of the vagus. Similar to Kim et al. [68], wild-type animals who received hemivagotomy prior to PFF injection had α-synuclein deposition only on the unvagotomized side, consistent with a vagal-dependent spread of pathology [70]. There were two groups that have also shown that oral delivery of α-synuclein fibrils can induce neurological symptoms and Lewy-like pathology in the CNS of heterozygous A53T α-synuclein transgenic mice [72,73]. Specifically, α-synuclein pathology was observed in the spinal cord, brainstem, and substantia nigra following oral administration of aggregated α-synuclein [72,73]. In addition, A53T transgenic mice were also found to harbor seeding-competent α-synuclein in colon tissue several months before similar species were detected in the brain, suggesting that pathological α-synuclein can form in the ENS prior to appearing in the CNS [74].

In some cases, gut-initiated α-synuclein spreading to the CNS was reported to be a transient phenomenon. Manfredsson et al. [75] injected rats and non-human primates with PFFs into the descending colon, and in both cases, observed abundant ENS pathology that persisted even after 1 year. In rats, minor α-synuclein pathology was also present in the DMN of the vagus and the locus coeruleus at 1-month post-injection, but this was not observed at later timepoints, and CNS lesions were not observed at any timepoint in the non-human primates. Since only ~20% of ENS ganglia in the descending colon receive vagal innervation [75], it is perhaps not surprising that little or no spread of pathology was observed in the brainstem of either the rats or monkeys. Moreover, the presence and then disappearance of the CNS lesions in this and other studies [70,71] argues that a certain threshold of α-synuclein transmission must be met in order to have sustained CNS pathology. Beneath this threshold, it is likely that cellular degradation mechanisms are able to clear the misfolded α-synuclein before further aggregation and spreading can occur.

4.4. C. elegans as a Powerful Model System to Study α-Synuclein Pathogenicity in PD

While rodent and non-human primate models provide essential information with regards to how α-synuclein can behave in a mammalian system, complementary animal models that offer a rapidly aging nervous system and high genetic tractability are

necessary to accelerate the discovery of disease mechanisms and potential treatments. The small nematode worm, *C. elegans*, provides such a platform, having a well-defined nervous system that gives rise to a complex set of behaviors [76], orthologs for 60–80% of human genes [77], conserved neurotransmitter signaling [78], and suitability to rapid large-scale behavioral and phenotypic screening approaches [76]. *C. elegans* is a premier model system to study aging and age-related disease, due to its short lifespan (2–4 weeks) and stereotyped age-dependent decline at the tissue, cellular, and molecular levels [79]. In addition, transgenic expression of human α-synuclein in worms has recapitulated progressive age-dependent neuron death, protein aggregation, and behavioral deficits [80–82], and proven useful for the study of cell autonomous disease mechanisms in dopaminergic neurons [83–85].

Several studies have also demonstrated cell–cell transmission of human α-synuclein in *C. elegans* [86–88]. Bimolecular fluorescence complementation (BiFC) is one technique that has been used to visualize α-synuclein transfer between cells. In this approach, α-synuclein in one group of cells is fused to the N terminus of a fluorophore, while α-synuclein in another group of cells is fused to the C terminus of the fluorophore. Thus, fluorescence should only occur if α-synuclein fusion proteins are able to translocate from one cell to another and come in sufficient proximity for the fluorophore to assemble. Using BiFC in *C. elegans*, α-synuclein has been shown to transfer from neuron–neuron in synaptically connected circuitry [86], and to travel bidirectionally between neurons and pharyngeal muscle cells [87]. The latter observation was associated with enhanced neurodegeneration, functional decline, and decreased lifespan, suggesting that α-synuclein transmission is toxic [87]. In another study, α-synuclein was observed to translocate from donor dopaminergic neurons or muscle cells to recipient hypodermis tissue [88]. While these findings indicate that cell–cell α-synuclein transmission can occur in *C. elegans*, the aggregation state of α-synuclein was often not evaluated, and critically, there remains a need for specifically gut-to-brain PD worm models.

In an effort to generate prion-like α-synuclein transmission models initiated in the gut of *C. elegans*, our group recently published the neurotoxic effects of feeding worms human α-synuclein PFFs [89]. To our knowledge, this is the first report of α-synuclein PFF exposure in *C. elegans*. Similar to mouse models, we found that PFF ingestion in *C. elegans* promotes dopaminergic neurodegeneration, accelerates the aggregation of host α-synuclein in muscle, and induces an age-dependent motor decline. Importantly, monomeric α-synuclein feeding was unable to produce the same effects, and PFF-fed worms lacking host α-synuclein were also protected [89]. These findings are consistent with prion-like propagation of disease by gut-derived α-synuclein fibrils, potentially via the worm alimentary nervous system, which remains to be tested. Our results are also consistent with previous reports of diet-induced α-synuclein aggregation in *C. elegans* [90]. Specifically, worms fed *E. coli* that produce the bacterial amyloid, curli, showed increased aggregation of α-synuclein in muscle cells. In the same study, aged rats that were fed curli-producing bacteria showed an increase in α-synuclein deposition in the ENS, as well as in the hippocampus and striatum [90].

To identify potential regulators of gut-to-brain synucleinopathy, our group used the rapid and highly manipulable *C. elegans* PFF models to conduct a targeted RNAi screen [89]. Previously, heparan sulfate proteoglycans (HSPGs) were identified as potential cell surface receptors for the internalization of α-synuclein PFFs in vitro [91]. We tested seven genes in the highly conserved HSPG pathway and showed that of those, five genes, namely *SDC1/sdn-1* (cell surface membrane-bound proteoglycan syndecan), *EXT1/rib-1* (exostosin glycosyltransferase 1), *EXTL3/rib-2* (exostosin like glycosyltransferase 3), *NDST1/hst-1* (N-deacetylase and N-sulfotransferase 1), and *HS3ST6/hst-3.2* (heparan sulfate-glucosamine 3-sulfotransferase 6), were necessary for PFF-dependent motor

dysfunction [89]. *SDC1/sdn-1*, *EXT1/rib-1*, and *NDST1/hst-1* were also required for PFF-induced host α-synuclein aggregation and dopamine neuron degeneration [89]. These results constitute the first in vivo evidence that HSPGs can regulate disease phenotypes caused by α-synuclein PFFs and suggest that the *C. elegans* PFF models can be used in future studies to further uncover the mechanisms of α-synuclein spread from the gut and resulting neurotoxicity.

4.5. Alternative Hypotheses of α-Synuclein Spreading in PD

Despite mounting evidence in humans and animal models supporting the gut-to-brain hypothesis of α-synuclein transmission in PD, alternative possibilities have been proposed that fuel ongoing debate. A major criticism of the gut-origin hypothesis of PD is the lack of individuals found to have α-synuclein pathology in the ENS in the absence of pathology in the CNS. It would be expected that if α-synuclein pathology begins in the ENS and spreads to the CNS via the vagal nerve, there should be normal subjects with undiagnosed, prodromal PD that harbor ENS and/or vagal nerve pathology without evidence of lesions in the CNS. To address this issue, Beach and colleagues [56] conducted an autopsy study of stomach and/or vagal nerve tissue from 111 normal elderly controls that had no CNS pathology, 33 ILBD cases with some CNS pathology, and 53 confirmed PD cases. None of the normal subjects were found to have α-synuclein lesions in the stomach or vagal tissue, whereas 17% and 81% of ILBD and PD cases, respectively, had stomach pathology, and 46% and 89% of ILBD and PD cases, respectively, had vagal pathology. The authors concluded that, once again, the lack of α-synuclein inclusions in the ENS/vagal nerve of normal control subjects argues against a gut-first hypothesis of PD, and instead supports a CNS origin of disease [56].

Several limitations of the study weaken this conclusion, however. If the gut-to-brain hypothesis is true, then the identification of ENS/vagal-positive α-synuclein cases among normal controls in a given study is predicated on the existence of prodromal PD cases within this group. The rate of PD among the aged (65 and older) population in the US is estimated to be 1.6% [92]. If this rate is also representative of prodromal PD in the US, then a sample size of 111 controls, as in the Beach et al. [56] study, would be expected to include at most only 2 subjects with ENS/vagal pathology (and no CNS pathology). Even with an estimated 90,000 new PD cases each year among 65 and older individuals in the US [93], and assuming a 20-year prodromal period [61], 1.8 million potential prodromal cases represent 3.2% of the 56 million people aged 65 and over in the US [94], corresponding to only 4 prodromal cases being included at most in the control group of the Beach study. Therefore, the low rate of prodromal PD estimated to exist in the aged population drastically reduces the probability of detection. Moreover, the Beach et al. [56] study only examined stomach tissue and did not look at other gastrointestinal organs, and similarly only sampled one area of the vagus nerve. These limitations notwithstanding, Beach and coauthors investigated an important question in a large human cohort, albeit probably not large enough to make firm conclusions regarding prodromal PD.

Rather than arguing specifically against the gut-to-brain hypothesis of PD, the discovery of pathological α-synuclein in the vagus nerve of the majority of PD patients and almost half of ILBD patients [56] can alternatively be interpreted as supporting the vagus nerve acting as a conduit for α-synuclein transmission between the gut and the brain, potentially in either direction. Consistent with this, Borghammer and colleagues have put forth the hypothesis that PD cases fall into two main categories based on the trajectory of α-synuclein spread: brain-first versus body-first [95]. In brain-first PD, it is postulated that α-synuclein aggregation begins in the olfactory bulb or amygdala and eventually spreads to the ENS via anterograde transmission through the vagal nerve. In body-first PD, pathological

α-synuclein arises first in the ENS and spreads to the CNS via retrograde transmission through the vagal nerve. Clinical evidence supports the grouping of PD patients into these categories [95], and animal studies have shown that α-synuclein is capable of travelling bidirectionally through the vagal nerve [96]. It is, therefore, likely that multiple subtypes of PD exist, making the probability of identifying prodromal body-first PD in normal control subjects even lower than predicted above.

5. Conclusions

Ample evidence now suggests that α-synuclein can act similarly to prions and can potentially infiltrate the CNS from peripheral tissues such as the gastrointestinal tract. It is likely that multiple sites of initiation exist for the primary α-synuclein misfolding event to occur, with subsequent amplification of pathological aggregates, escape into the extracellular space, and infection of neighboring cells within synaptically connected networks. The vagal nerve offers a direct route by which α-synuclein may be able to propagate from the ENS to central brain regions, and both human and animal studies support this pathway as a prime candidate for PD progression.

Critical questions remain to be answered, however. At the level of the ENS, it is still unknown which cell types contain α-synuclein inclusions and could theoretically transmit pathological species through interconnected circuitry. Although VIPergic neurons were originally identified as a major cell type harboring LBs in PD [12], this finding was not reproduced in a later study [18]. Conflicting data also exist regarding potential enteric neuron degeneration in PD. At least two studies have found enteric ganglion cell degeneration [9,22], while several others have reported no evidence of neuronal cell loss in the ENS [11,12,18]. In addition, a major outstanding question is what factors might initially trigger α-synuclein misfolding in the ENS. Braak et al. [97] originally proposed that an unknown pathogen in the gut, such as a virus or prion particle, could induce α-synuclein aggregation in enteric neurons. This kind of pathogen, potentially α-synuclein itself introduced from the diet [98], might be able to gain entry across the gut epithelial barrier under conditions of increased permeability, such as "leaky gut" caused by inflammation or infection [98].

Another critical question is whether ENS pathology can be used as a biomarker for the early detection of PD. The discovery of a reliable biomarker in pre-Parkinsonian patients would be highly clinically significant, allowing for the potential of an early intervention that could slow or even prevent further disease. Despite extensive research efforts, the utility of α-synuclein detection in gastrointestinal biopsies and surgical resections remains unclear [99]. Methodological issues have posed great challenges, including the insufficient sensitivity and specificity of some α-synuclein immunohistochemical approaches for distinguishing PD patients from controls [21]. Still, there are studies that show promising results, such as a recent report of duodenal biopsies examined using the conformation-specific 5G4 α-synuclein antibody that preferentially detects aggregates [100]. This study found greater α-synuclein aggregation in PD cases compared with controls and detected pathology in both early- and late-stage PD patient biopsies [100].

A depiction of the gut-to-brain hypothesis of PD highlighting these and other unresolved questions can be found in Figure 3. The development of new animal models, such as gut-to-brain PD models in *C. elegans* [89], and new technologies including gut- and brain-specific organoids [101] may serve to complement existing rodent model systems by acting as platforms for high-throughput discovery. In all, the theory of gut-to-brain transmission of α-synuclein in PD is supported by a compelling body of evidence and warrants further study to determine its precise clinical relevance.

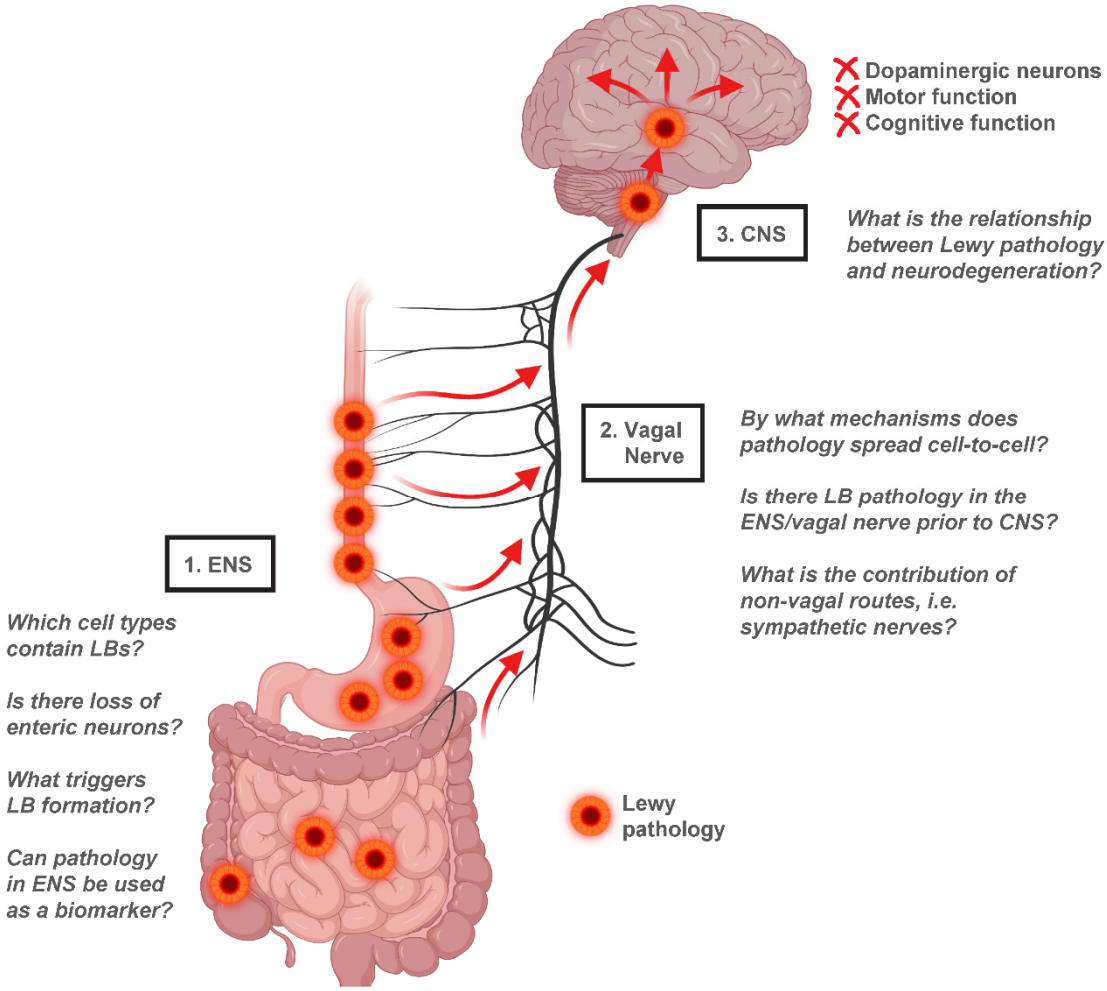

Figure 3. Gut-to-brain α-synuclein transmission hypothesis of PD. Evidence from human studies suggests that Lewy pathology in the ENS of PD patients is concentrated in the upper gastrointestinal tract, with the greatest LB density found in the esophagus and stomach. This distribution parallels vagal innervation of the ENS, consistent with a potential transmission of α-synuclein from the ENS retrogradely through the vagal nerve into the CNS. Once in the CNS, Lewy pathology appears to spread from the brainstem to the midbrain and finally to cortical regions, resulting in neurodegeneration and functional decline. Several unresolved questions relating to the gut-to-brain hypothesis of PD are highlighted. Figure created using Biorender.com.

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Manuscript in Preparation: Treatment of *C. elegans* with α-synuclein pre-formed fibrils to induce Parkinson's-like disease

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Introduction

Parkinson's disease (PD) is a debilitating neurological disorder that impairs aging individuals' motor function as the disease progresses. PD is characterized by and diagnosed port-mortem by the identification of large aggregates of the misfolded protein α-synuclein (α-syn) in brain tissue, which in healthy individuals remains in monomeric or oligomeric form. It is unclear precisely how aggregation is initiated, and whether aggregates themselves interact with neurons to accelerate and/or promote neuronal degeneration and death, or if they are simply the most visible symptoms of less well-understood disease mechanisms. Recent studies have identified a possible gut-to-brain link in PD patients wherein protein aggregation is observed first in the gut and later detected in the brain: indeed, early symptoms of PD include gastrointestinal changes and differences in gut microbiota compared to healthy individuals. Model systems in which human wild-type α-syn can be expressed present promising research opportunities for uncovering the mechanisms of α-syn aggregation and the possibility of α-syn misfolding originating in the gut. *C. elegans* remains a vital model organism in the study of neurodegeneration for its high throughput testing capabilities, rapid lifespan, and fully mapped neuronal network, enabling the precise identification of degenerating neurons, to rapid motor and chemical assays involving thousands of individuals. Along with its ease of imaging, *C. elegans* may be fed specific protein and bacteria diets, which can elucidate how α-syn protein deposition in the gut may seed and/or accelerate the progression of the disease and neuronal death elsewhere in the body. Though a number of studies have been performed on how endogenous expression of human wild type α-syn can alone cause neurodegeneration in worms as they age, only one study to date has paired feeding pre-formed α-syn fibrils (PFFs) with α-syn-expressing worms to observe how exogenous α-syn may seed the misfolding of endogenous α-syn within the worm body. We have developed a PFF-feeding protocol to enable researchers to test the gut-to-brain seeding and aggregation effects of α-synuclein in *C. elegans* for both small-scale and large-scale experiments and have provided representative data for motor assays of the treated worms.

Reagents

- α-syn monomer (We used Proteos® RP-003 "Human Alpha-synuclein monomer to generate preformed fibrils" 10 mg/mL in 10mM Tris, 50mM NaCl, pH 7.6)
- PBS pH 7.4 (1X) filtered through a 5-µm membrane
- M9 buffer (KH_2PO_4, NA_2HPO_4, NaCL, $MgSO_4$ (1M))

Equipment

- Pipettes: 10, 200, and 1000-µL
- Sterile filtered pipette tips (regular and large orifice for 200-µL)
- -80°C freezer
- 20°C fridge
- Sterile 1.5-mL microcentrifuge tubes
- Mini centrifuge for 1.5-mL tubes
- Thermomixer with block for 1.5-mL tubes
- BSL-2 culture hood
- 30-mL sterile syringe
- 5.00µm syringe-driven filter unit
- Untreated normal growth media plates
- Untreated high growth media plates
- OP50-seeded high growth media plates
- 15-mL high-clarity polyproplylene conical tubes
- Serological pipettes: 10-mL
- Pipettor
- 50-mL centrifuge tubes
- Ecolab® Isolyser™ Isosorb™ liquid treatment system for medical waste encapsulation

Procedures

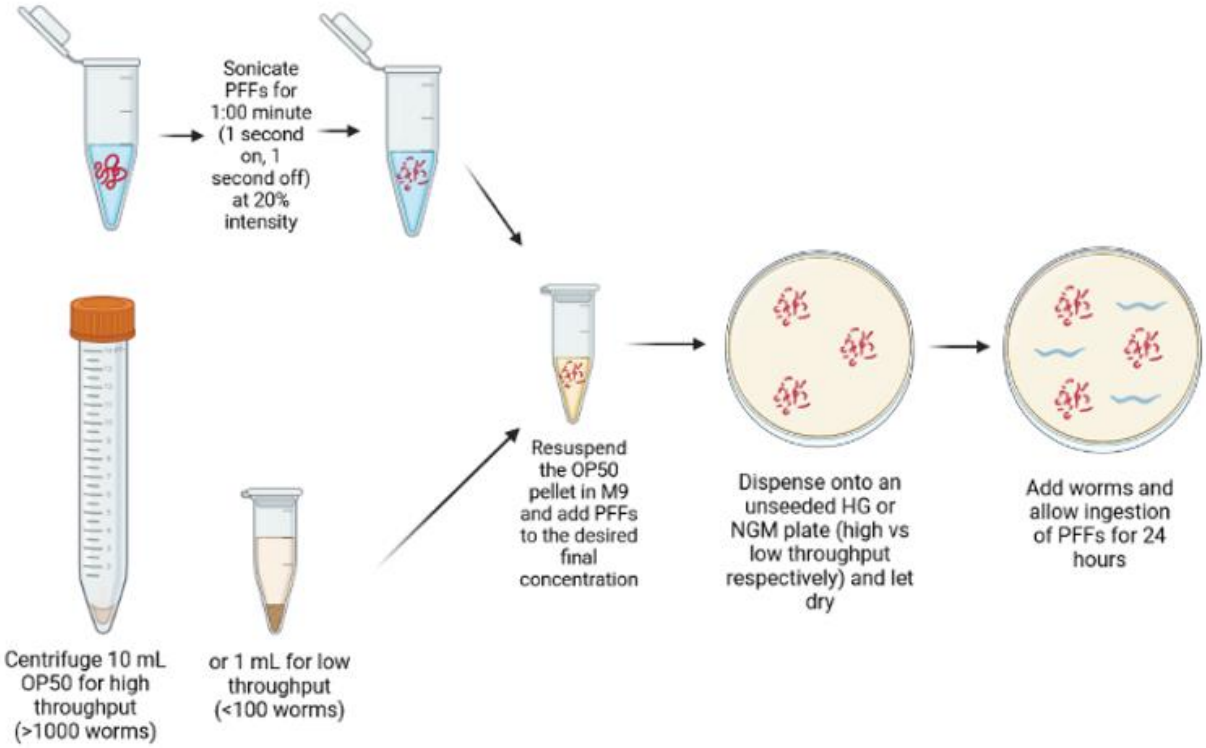
PFF aggregation

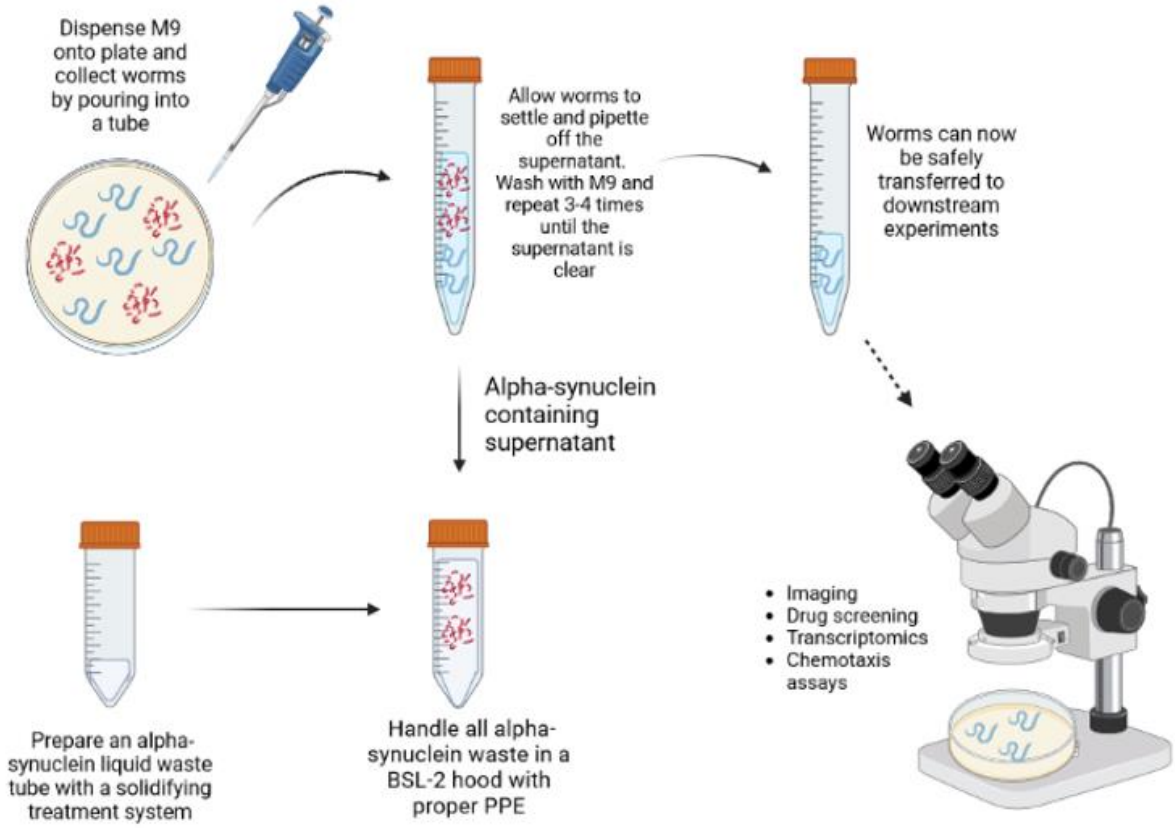
1. Completely thaw the α-syn monomer in a 1.5-mL tube on ice.
2. Spin down the tube in a mini centrifuge to remove any droplets from the lid.
3. Add filtered PBS to the tube to a final concentration of 6-mg/mL and mix by pipetting slowly up and down.
4. Transfer the solution to a new 1.5-mL microcentrifuge tube. Close the tube tightly and wrap parafilm around the lid to prevent any liquid from escaping during the shaking stage.
5. Place the tube in a Thermomixer and ensure that a plastic lid or foil is secured and covering the heating block. Start the mixer at 37°C at 1400 rpm and run for 7 days.
6. Carefully remove the tube from the thermomixer and spin down to collect condensation from the lid.

7. In a BSL-2 hood, gently pipette the solution up and down to mix. It is highly recommended to wear two layers of gloves whenever working with PFFs, in addition to a lab coat with sleeves that completely cover the wrists.
8. Carefully aliquot the PFFs into separate labeled 1.5-mL microcentrifuge tubes. Store the aliquots at -80°C. PFFs will dissociate at higher temperatures.

Preparation of PFF- and monomer-containing food source for C. elegans

1. Completely thaw one aliquot each of the prepared PFFs and monomers: the monomer tube must be thawed on ice, and the PFF tube may be thawed at room temperature.
2. Spin down the tubes to collect any droplets from the sides and add at least 200 µL of filtered PBS to each tube, taking note of the final concentration. Keep the monomer tube on ice until it is used.
3. Place the PFF tube in a small beaker of ice that can easily be held upright by hand. Set the sonicator to run for 1:00 minute at 1 second on, 1 second off, for a total of 2 minutes at 20% intensity. Sonicate the PFFs.
4. Count out as many empty 1.5-mL microcentrifuge tubes as the number of treatment plates that will be used for the experiment.
    a. For high-throughput experiments, multiple plates will be necessary for each treatment group to ensure that worms do not starve.
5. For small-scale experiments, pipette 1 mL of OP50 into each of the empty tubes and pellet the bacteria by centrifuging at 5000g for 5 minutes. Remove all supernatant.
    a. For high-throughput experiments, pipette 10 mL of OP50 each into 15-mL conical tubes. Centrifuge at 3500g for 5 minutes and remove all supernatant.
6. Determine the volumes of M9 buffer and PFFs or monomers to add to each bacteria tube for the desired treatment concentration. M9 buffer is added first to resuspend the bacteria pellet until it is fully dissolved, and then the PFFs or monomers are added, pipetting gently up and down to mix.
    a. For high-throughput experiments, dissolve the bacteria in the 15-mL tube in M9 buffer first and then transfer the required volume into the 1.5-mL tube before the PFFs or monomers are added.
7. Dispense the control, monomer, or PFF solution from each bacteria tube onto a correspondingly labeled normal growth media plate for small-scale experiments, or high growth media plate for high-throughput experiments. Gently swirl the plate and allow it to dry.


Sonicate PFFs for 1:00 minute (1 second on, 1 second off) at 20% intensity
Centrifuge 10 mL OP50 for high throughput (>1000 worms)
or 1 mL for low throughput (<100 worms)
Resuspend the OP50 pellet in M9 and add PFFs to the desired final concentration
Dispense onto an unseeded HG or NGM plate (high vs low throughput respectively) and let dry
Add worms and allow ingestion of PFFs for 24 hours


Dispense M9 onto plate and collect worms by pouring into a tube
Allow worms to settle and pipette off the supernatant. Wash with M9 and repeat 3-4 times until the supernatant is clear
Worms can now be safely transferred to downstream experiments
Alpha-synuclein containing supernatant
Imaging
Drug screening
Transcriptomics
Chemotaxis assays
Prepare an alpha-synuclein liquid waste tube with a solidifying treatment system
Handle all alpha-synuclein waste in a BSL-2 hood with proper PPE

Worm treatment and safe transfer for downstream assays

Note: Worms should be transferred onto the prepared PFF- and monomer-containing food sources as soon as the plates are dry. It is not recommended to prepare and stockpile treatment plates in advance, as the structure of the PFFs and monomers may change the longer they remain above -80°C.

- Worms may be picked directly onto the treatment plates for small-scale experiments. No further preparation or precautions are necessary.
    1. For high-throughput experiments, wash day 1 adult worms off high-growth plates with 2-3 mL M9 buffer, pouring carefully into a 15-mL tube.
    2. Allow the worms to settle for 2-3 minutes and then vacuum off the supernatant. Add up to 5 mL of M9 to the tube, repeat at least two times until the supernatant is clear.
    3. Remove the remaining supernatant, leaving the worm pellet undisturbed, and use a large orifice pipette tip to transfer up to 30 µL of worms per 10X OP50 onto each of the treatment plates. It is not recommended to exceed this ratio, as the worms may starve by the following day.
    4. Store the plates in a 20°C fridge and allow the worms 24 hours of exposure to the treated food source.
- For recovery of the worms, handle all plates in a BSL-2 hood, as the process produces α-syn liquid waste. Adhere to all PPE requirements.
    5. Prepare and label multiple 50-mL waste tubes by adding 2.5 g of Ecolab® Isosorb™ into each for the collection of α-syn liquid waste. 2.5 g of the product will safely absorb and solidify 50 mL of solution.
    6. Wash the worms off their plates in M9 buffer and carefully pour into labeled 15-mL tubes for each treatment group.
    7. Allow the worms to settle for 2-3 minutes. Carefully pipette off the supernatant with a micropipette, dispensing it into the labeled α-syn waste tube. Supernatant for the control group may be vacuumed into a flask and treated as OP50.
    8. Add up to 5 mL of M9 to each tube, allow the worms to settle, dispense the supernatant, and repeat until the supernatant is clear. It is crucial that no α-syn-containing bacteria is transferred downstream in the experiment.
- At this stage, the worms may be moved onto new high-growth media plates or used immediately in an experiment.

Motor assays of PFF- and monomer-treated worms in WormLab®

WormLab® is a video analysis software developed by MBF Bioscience that tracks movement of C. elegans in recorded video and produces motor data such as center point speed, amplitude, and wavelength. It and other worm tracking software can identify potentially overlooked motor

phenotypes that are not described by standard body bends per time assays but are crucial in studying neurodegeneration.

We captured 30-second videos of PPF, monomer, and buffer-treated worms on unseeded NGM plates as .mp4 files. Worms were picked with OP50 and moved and recorded individually, as our camera's field of view was limited. Recording began within 5-10 seconds of worm placement, and after recording, worms were returned to their original plate and could be used again for video capture the following day.

Representative results

Adult wild type α-syn worms exhibit significant differences between treatment groups for specific motor parameters beginning on day 3 of adulthood. We tested straight line distance, turn count (forward body bends), reversal distance, reversal time, number of reversals, maximum amplitude, mean amplitude, mean wavelength, and center point speed.

One-way ANOVA testing between the buffer treatment and either the monomer- or PFF-treated worms did not yield any significant differences on day 2 of adulthood. On day 3, mean wavelength, mean amplitude, and center point speed became significantly different between the buffer and PFF-treated groups while the monomer treatment remained unchanged.

We also tested non-α-syn-expressing, GFP-labeled BY250 worms at the same time points with the same treatment parameters and found no significant differences in motor function on day 3.

One point of clarification in the lack of significant results for turn counts is that the software does not appear to track bends made in reverse: our previous study and manual motor assays performed in conjunction with the worm videos continued to show differences in body bend counts between buffer and PFF-treated worms when reversal bends were included.

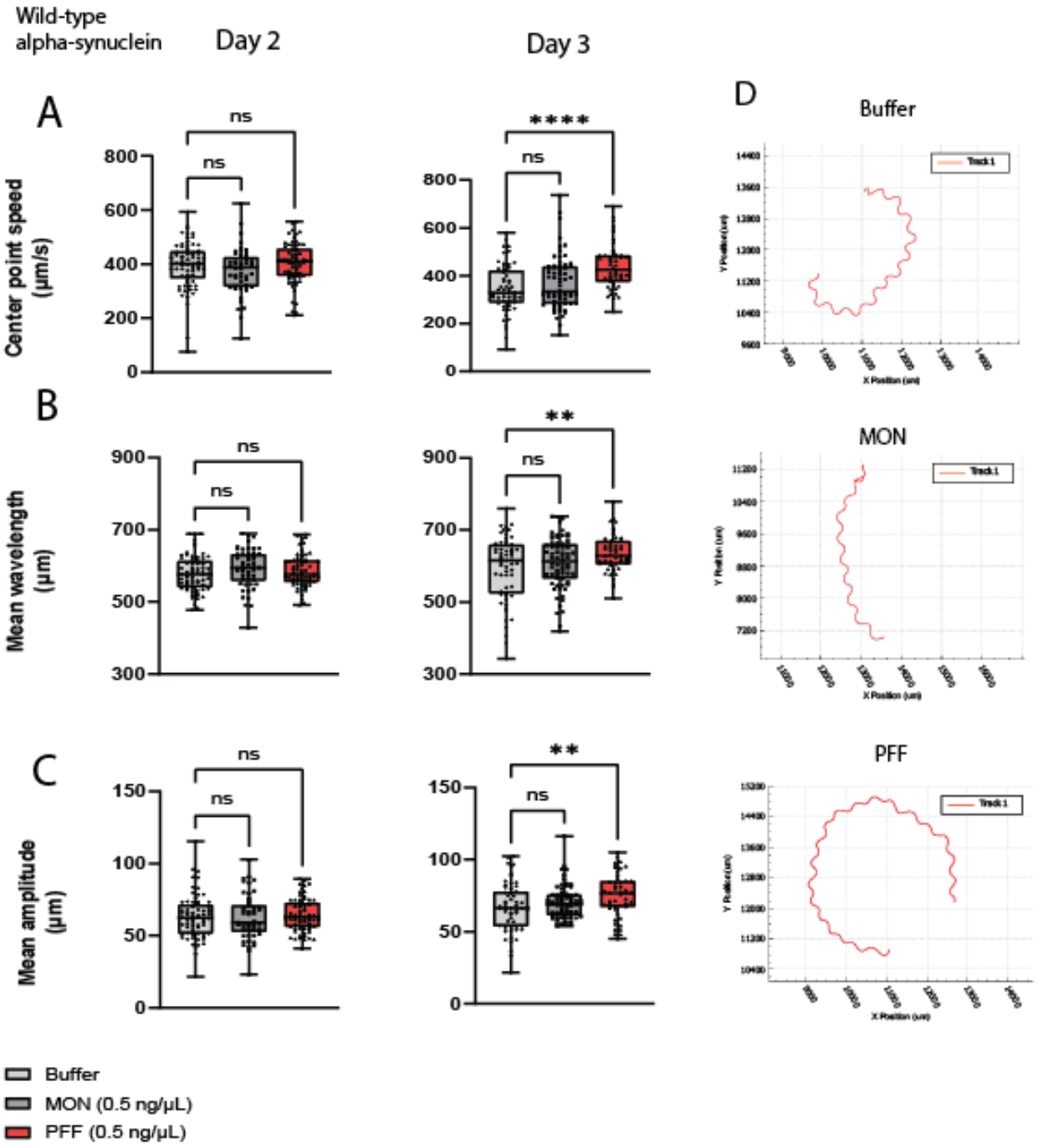


Figure 2. Motor metrics of buffer, monomer, and PFF-treated wild-type α-syn-expressing worms on days 2 and 3 of adulthood with representative worm traces. All significant effects became apparent on day 3 of adulthood. The PFF-treated worms exhibited **A)** a higher average center point speed (µm/s, p value < 0.0001), **B)** higher mean wavelength (µm, p value = 0.0047), and **C)** higher mean amplitude (µm, p value = 0.0073) than the buffer-treated worms. There were no significant differences between the

buffer-treated and monomer-treated worms. Each treatment group tested between 50-70 worms pooled from three replicates. **D)** Worm traces provide a visual representation of differences in wavelength and amplitude, more notably the greater wavelength as evidenced in the PFF treatment.

Discussion

The motor differences we observed in PFF-treated α-syn-expressing worms suggests that the worms begin to shift to a new pattern of locomotion as protein aggregation accelerates. They exhibit greater average scores in both wavelength and amplitude, creating worm paths that are visually and statistically distinct from buffer-treated worms. These differences in movement, i.e. wider and longer turns, indicate that the worms may progressively lose their ability to make tight, short turns---mirroring the loss of fine motor control observed in human PD patients.

Interestingly, the PFF-treated worms also demonstrated faster center point speeds, which ultimately compensated for their longer-than-average turns and resulted in equal distances traveled between the treatment groups. This shift toward larger turns also validates previous findings from our manual body bend assays, in which PFF-treated worms made fewer average turns despite appearing to travel the same distance as the other groups.

With the low and high throughput treatment protocols, worms affected by gut-initiated α-syn aggregation can be tested for motor defects, learning and memory retention, and other markers for PD-related decline. High throughput treatment is especially promising for assessing drug efficacy, as large numbers of worms can be screened for neuron and motor recovery through automated image capture and analysis pipelines.

Additional Figures

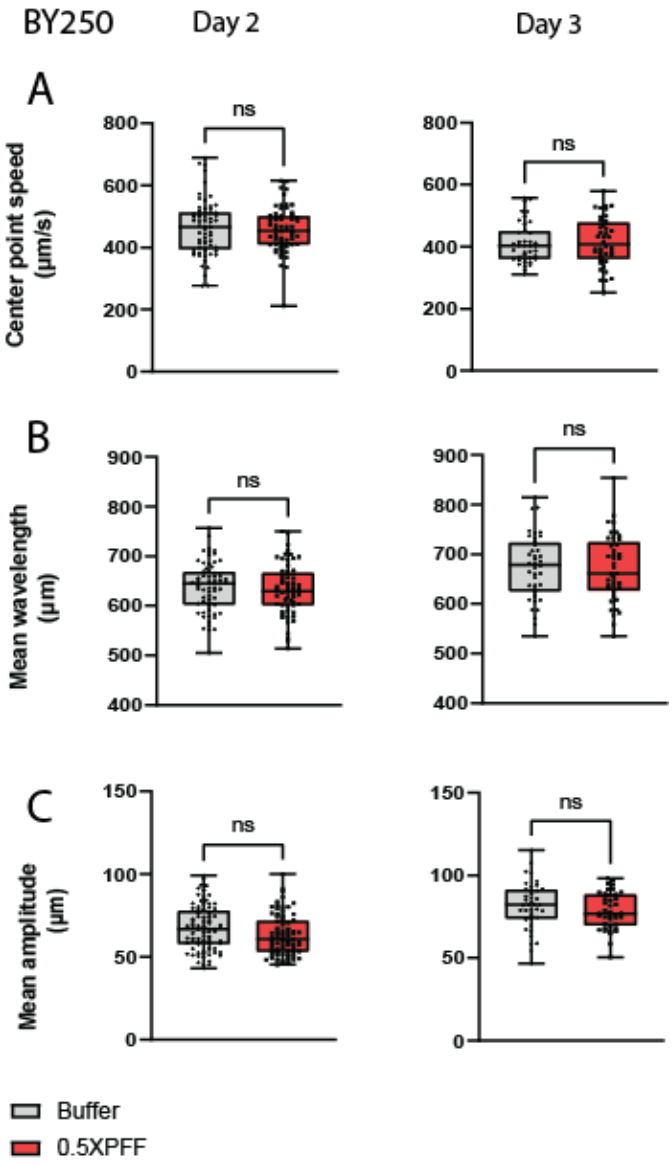


Figure 3. Motor metrics of buffer and PFF-treated BY250 GFP-expressing worms on days 2 and 3 of adulthood. No significant differences were identified in any metrics on either day. Two replicates were pooled with 15-20 worms each.