AWARD NUMBER: W81XWH-16-1-0676

TITLE: Defining the Role of Alpha-Synuclein in Enteric Dysfunction in Parkinson's Disease

PRINCIPAL INVESTIGATOR: Fredric P Manfredsson

CONTRACTING ORGANIZATION: Michigan State University

REPORT DATE: Jan 2020

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE
Jan 2020

2. REPORT TYPE
Final

3. DATES COVERED
09/15/2016 - 09/14/2019

4. TITLE AND SUBTITLE
Defining the Role of Alpha-Synuclein in Enteric Dysfunction in Parkinson's Disease

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-1-0676

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Fredric P Manfredsson

E-Mail:fredric.manfredsson@hc.msu.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Michigan State University
426 Auditorium Rd
Rm 2
East Lansing, MI 48824

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The most recognizable feature of Parkinson's disease (PD) is a set of well-defined motor symptoms that arise due to the loss of neurons in the substantia nigra (SN). Nevertheless, it has become increasingly recognized that PD patients also suffer from a plethora of non-motor symptoms. Of those symptoms, gastrointestinal (GI) dysfunction is often described as extremely debilitating. Moreover, GI dysfunction can also complicate symptomatic management of the disease. Importantly, the same pathology that can be seen in the SN is also observed in the enteric nervous system (ENS), the network of neurons that control GI function. In order to better study the role of this pathology; aggregation of the protein alpha-synuclein (α-syn) in enteric neurons, we devised a gene therapy method aimed at directly delivering a pathological dose of α-syn to the ENS per se. This approach allows us to directly study the role of (pathological) α-syn in the ENS, without the confound of inducing pathology in other neuronal populations. Our overarching hypothesis stated that that the pathological presence of aggregated α-syn in the ENS impedes neuromuscular transmission responsible for propulsive colonic motility. We further proposes that colonic motility and contractility will progressively decrease over time, without any overt neurodegeneration of the ENS as is the case with human disease. In year 1 we observed that 1) Low level of ectopic α-syn overexpression in enteric neurons results in impaired contractility of the colon, and 2) This reduction in contractility is associated with a reduction of colonic motility.

15. SUBJECT TERMS
Parkinson's disease, synucleinopathy, alpha-synuclein, gene therapy, viral vector, enteric nervous system, Lewy body, Lewy pathology, gastrointestinal, gastric dysmotility, constipation, prion spread.

16. SECURITY CLASSIFICATION OF: U			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U Unclassified	**b. ABSTRACT** U Unclassified	**c. THIS PAGE** U Unclassified	UU Unclassified	19	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Page

1. **INTRODUCTION:**

The most recognizable feature of Parkinson's disease (PD) is a set of well-defined motor symptoms that arise due to the loss of neurons in the substantia nigra (SN). Nevertheless, it has become increasingly recognized that PD patients also suffer from a plethora of non-motor symptoms. Of those symptoms, gastrointestinal (GI) dysfunction is often described as extremely debilitating. Moreover, GI dysfunction can also complicate symptomatic management of the disease. Importantly, the same pathology that can be seen in the SN is also observed in the enteric nervous system (ENS), the network of neurons that control GI function. In order to better study the role of this pathology; aggregation of the protein alpha-synuclein (α-syn) in enteric neurons, we devised a gene therapy method aimed at directly delivering a pathological dose of α-syn to the ENS *per se*. This approach allows us to directly study the role of (pathological) α-syn in the ENS, without the confound of inducing pathology in other neuronal populations. Our overarching hypothesis stated that the pathological presence of aggregated α-syn in the ENS impedes neuromuscular transmission responsible for propulsive colonic motility. We further proposed that colonic motility and contractility will progressively decrease over time, without any overt neurodegeneration of the ENS as is the case with human disease. We proposed to test this hypothesis in 2 complementary Specific Aims: 1) α-syn overexpression and aggregation within enteric neurons of the colon causes a progressive decline in colonic motility that is NOT associated with ENS neurodegeneration and 2) α-syn overexpression in the ENS causes GI dysfunction by decreasing excitatory neuromuscular transmission in the colon.

2. **KEYWORDS:**

Parkinson's disease, synucleinopathy, alpha-synuclein, gene therapy, viral vector, enteric nervous system, Lewy body, Lewy pathology, gastrointestinal, gastric dysmotility, constipation, prion spread.

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.*
   - **What were the major goals of the project?**
     - Animal use approvals – accomplished pre-funding
     - Vector production - 1st round of vector production was accomplished in month 1. A second round of vector production with modified genomes (see problem section below) was accomplished in July/August 2017. 100% Complete
     - Vector injections. We injected all animals for the long-term survival group as well as additional subjects for shorter time points. However, as noted below, the transgene expression seen in these animals was below that which was expected/intended. Thus, we repeated all injections in a new cohort of animals with a new vector.
     - We have performed *in vivo* colonic motility assay on all animals in the long term group.
     - We have performed *ex vivo* colonic assays on a subset of animals

- We performed *ex vivo* force transduction assays and histological analysis of some animals in shorter time points. Many of these outcomes have now been repeated in some of the new animals
- We have performed histological analyses in the long-term groups.

o **What was accomplished under these goals?**

As outlined in the original SOW, animals in the longest survival time underwent monthly evaluation using the bead expulsion assay. As expected α-syn overexpression was associated with a significant delay in bead expulsion, i.e. α-syn treated subjects exhibit colonic dysmotility (Figure1).

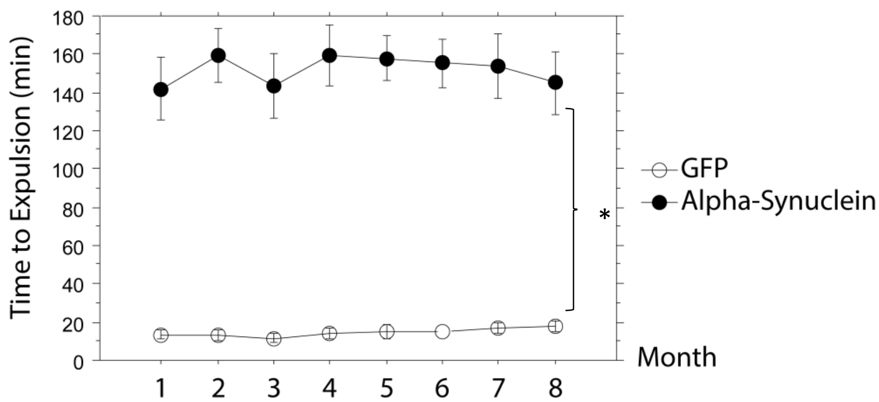

Figure 1. Assessment of in vivo colonic motility using the bead assay. *AAV-α-syn treatment (black circles) was associated with significant slowing of colonic motility as compared to AAV-GFP treated animals (n=6-16; * p<0.001). No significant change in motility was observed with either treatment over time.*

However, upon sacrifice of initial cohorts of animals we immediately observed that transduction was much lower than anticipated (*see* Figures 2 & 3).

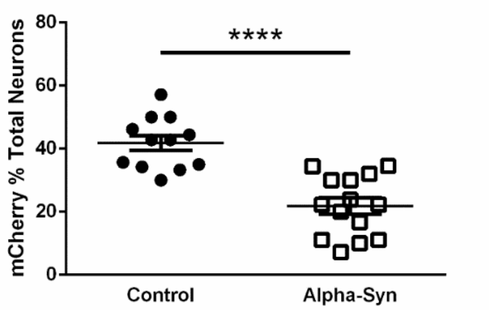

Figure 2. Quantitation of the mCherry reporter. *Animals received a bicistronic AAV expressing both mCherry and α-syn or BFP2. Quantitation of mCherry+ cells revealed that α-syn overexpression was associated with reduced mCherry expression. Ongoing studies are evaluating whether this is a due to neurodegeneration. * p<0.01.*

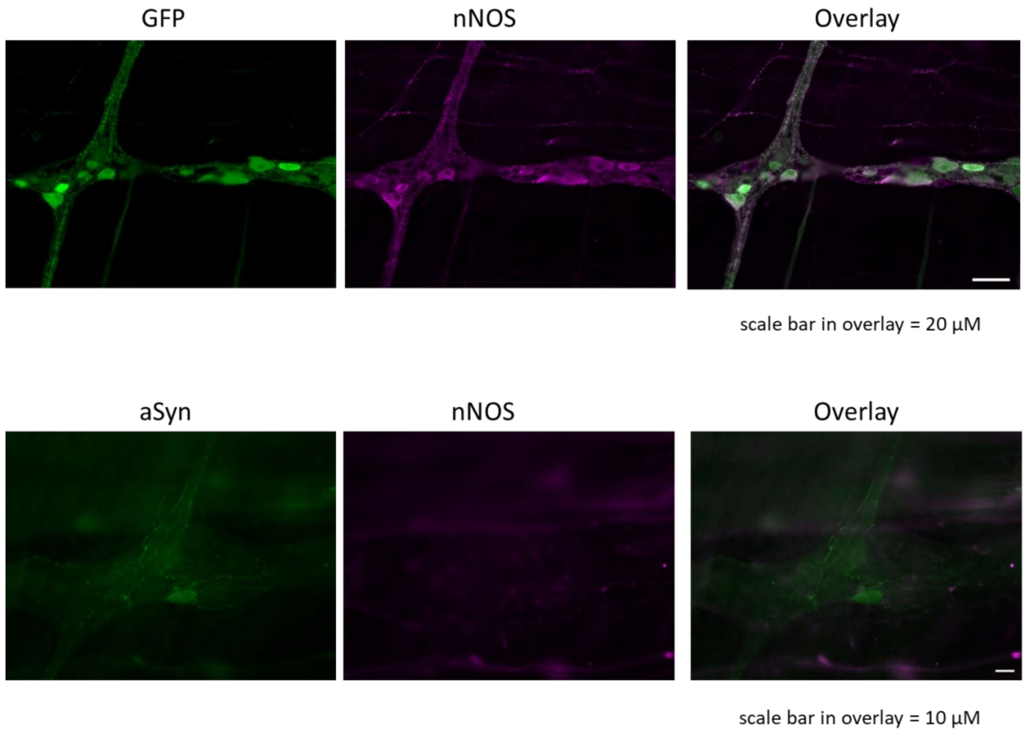

Figure 3. Initial assessment of transduction efficacy of control vector (top panels) or aSyn (lower panels). *It is clear that aSyn immunoreactivity is very weak (in contrast to the GFP control transgene).*

This finding was surprising given the fact that α-syn-treated animals exhibited a significant reduction in colonic dysmotility despite the low level of α-syn expression. It is not entirely clear what caused the reduction in transduction efficacy (titers were normalized as described in the original proposal to $1x10^{12}$ vector genomes (vg)/ml). Although we are now investigating this in a separate, unrelated, study we do think that the incorporation of two similar promoters resulted in genomic instability and recombination. We therefore repackaged the genome to contain a single cistron (pCBA + α-syn or GFP). As can be seen in Figure 4, this vector is producing much stronger transgene levels using the same dose.

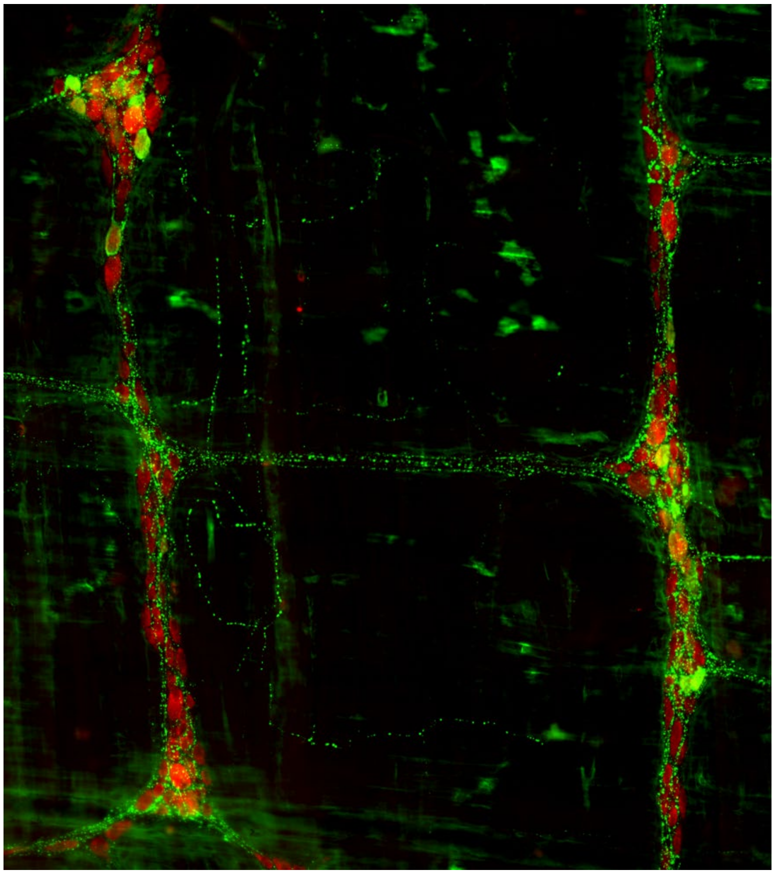

Figure 4. Transduction following delivery of rebuilt vector. *The α-syn vector was delivered using the exact parameters described in the proposal and as that used in animals shown in Figure 2. Robust α-syn (green) expression can be seen throughout the neurons (HuC/D; red) and their projections.*

α-syn overexpression is associated with loss of neurons of the myenteric plexus.

In addition, we postulated that one explanation for the observed changes in transduction was that α-syn overexpression could cause overt neuronal loss (which occurs in the analogous model in the CNS). Accordingly, whole mount preparations from rat colon treated with AAV-α-syn or GFP (sacrificed 6 months post-vector delivery) were stained for HuC/D (pan neuronal marker) and the total number of neurons were manually counted (> 5 ganglia per area, 3 areas (0.5 x 0.5mm) per subject). Interestingly, α-syn overexpression was associated with a significant loss of total myenteric neurons (**Figure 5**). In ongoing analyses we will 1) Add additional animals to the analysis, and 2) Assess if a specific neuronal population (e.g. DA neurons) is particularly susceptible to α-syn overexpression. Moreover, neurodegeneration is not reported in the human population, and because many of our functional outcomes were conducted also at two months post-vector delivery, we will also evaluate neuronal survival at additional (earlier) time points.

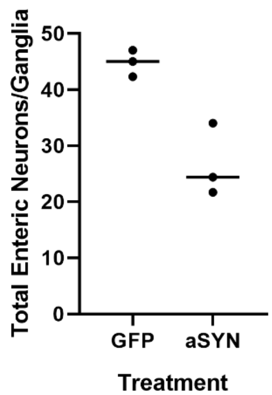

Figure 5. α-syn overexpression is associated with degeneration of myenteric neurons
*Animals received AAV expressing either GFP or α-syn and animals were sacrificed ~6 months later. AAV-α-syn was associated with significantly reduced fewer HuC/D+ neurons/myenteric ganglia. * $p<0.05$.*

Telemetry

We have completed all telemetry experimentation. Although preliminary data suggested that we would see an effect over time, no significant difference in telemetry was observed between either group (data not shown).

Ex vivo analysis

α-syn overexpression is associated with alterations in enteric muscle inhibition junction potential (IJP). Subjects received injections with either AAV into the proximal colon. Following sacrifice, preparations of circular muscle were utilized for IJP recordings. Overall, no difference in IJP amplitude was observed with α-syn overexpression (Figure 6A). However, an overall decrease in activity was detected in AAV-α-syn treated subjects (Figure 6B). The same measurements were done in the presence of MRS2179 (purinergic G protein-coupled receptor P2Y1 (P2Y1R) antagonist). Again, there was no effect of α-syn overexpression on IJP amplitude, but overall IJP activity was reduced in α-syn overexpression subjects (Figure 7). Finally, both treatment groups showed similar changes in IJP amplitude and activity with P2Y1 inhibition (Figure 8). These findings thus far suggest that α-syn overexpression does not affect purinergic neurotransmission (fast component of the IJP), but may interfere with nitrergic (nitric oxide, non-vesicular) neurotransmission (slow component of the IJP).

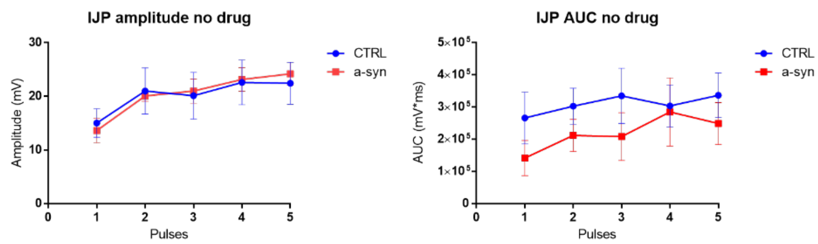

Figure 6. α-syn overexpression is associated with overall reduction in IJP. *Subjects were injected with either AAV-GFP (blue) or AAV-α-syn (red) into the proximal colon. Following sacrifice the targeted area was prepared for IJP recordings of circular muscle.* ***Left:*** *No difference in IJP amplitude was observed; however, α-syn overexpression was associated with lower overall IJP activity (as measured by AUC;* ***right****).*

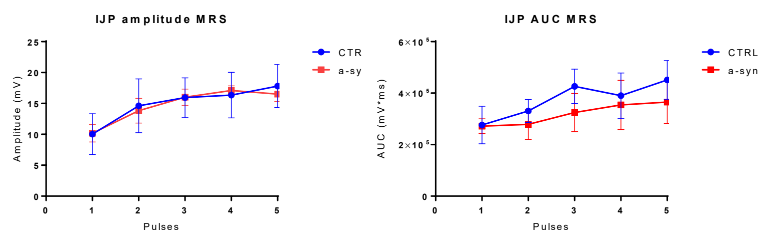

Figure 7. The effect of α-syn on IJP activity is not affected by Inhibition of purinergic receptors. *Subjects were injected with either AAV-GFP (blue) or AAV-α-syn (red) into the proximal colon. Following sacrifice the targeted area was prepared for IJP recordings of circular muscle which were performed in the presence of MRS2179.* ***Left****. No difference in amplitude was observed between treatment groups in either IJP amplitude. However, overall activity was reduced in animals receiving AAV-α-syn (****right****).*

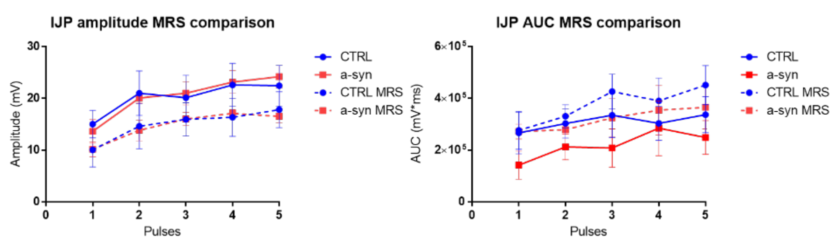

Figure 8. Treatment groups show the same response *Subjects were injected with either AAV-GFP (blue) or AAV-α-syn (red) into the proximal colon. Following sacrifice the targeted area was prepared for IJP recordings of circular muscle.* ***Left:*** *α-syn and control treated animals showed a similar decrease in IJP amplitude, and the same overall increase in IJP activity with P2Y1 antagonism.*

<u>Colonic migrating motor complex (CMMC).</u>

Our preliminary data, and data collected in previous years, suggested a significant effect on colonic motility and contractility due to α-syn overexpression. In order to further elucidate the underlying mechanism, we also performed measurements of CMMC. We observed that α-syn overexpression was associated with a significant decrease in CMMC velocity, but not frequency (**Figure 9**).In order to determine whether the degree of ectopic α-syn corresponds to the observed phenotype, in upcoming post-hoc analyses we will measure the degree of (α-syn) overexpression and aggregation in tissue utilized for *ex vivo* measures such as this.

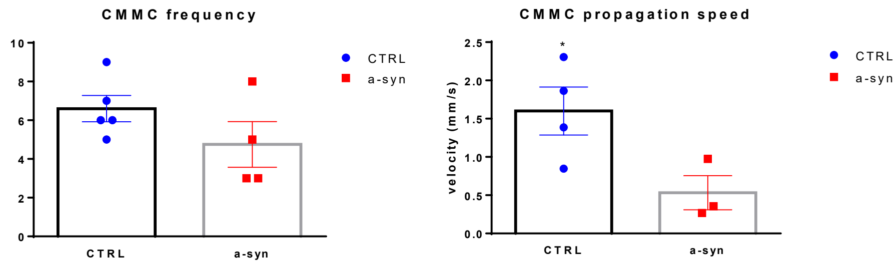

Figure 9. α-syn overexpression is associated with changes in the colonic migrating motor complex. *Mice were injected with either AAV-GFP (blue) or AAV-α-syn (red) into the proximal colon. 2 months following the vector delivery, the targeted area was prepared for spontaneous CMMC recordings.* ***Left:*** *No difference in frequency was observed; however, α-syn overexpression was associated with a significantly slower propagation of CMMC along the colon (**right**).*

Neurogenic contractions. Circular muscle contractions evoked by trains (1 s duration, 0.1 -20 Hz) of transmural electrical stimulation were studied in the absence and presence of drugs. There were no differences in amplitude by tissues from GFP or a-Syn treated rats (Fig. 6A,B). The muscarinic cholinergic receptor antagonist scopolamine (1 mM) reduced neurogenic contractions in colon tissues from GFP and a-Syn treated rats indicating that the contractions were largely mediated by acetylcholine (Fig. 7A,B). The N-type calcium channel blocker w-conotoxin (CTX, 0.1 mM) reduced the amplitude of contractions in tissues from GFP treated rats but this change was not statistically significant (Fig. 7C). CTX did reduce significantly the amplitude of contractions in tissues from a-Syn-treated rats (Fig. 7D). The voltage-gated sodium channel blocker, tetrodotoxin (TTX, 0.3 mM) did not inhibit the electrically evoked contractions in GFP or a-Syn treated tissues (Fig. 8C). Likewise, the nitric oxide synthase (NOS) inhibitior, nitro-L-arginine did not alter contraction amplitude in tissues from both groups of rats (Fig 8D).

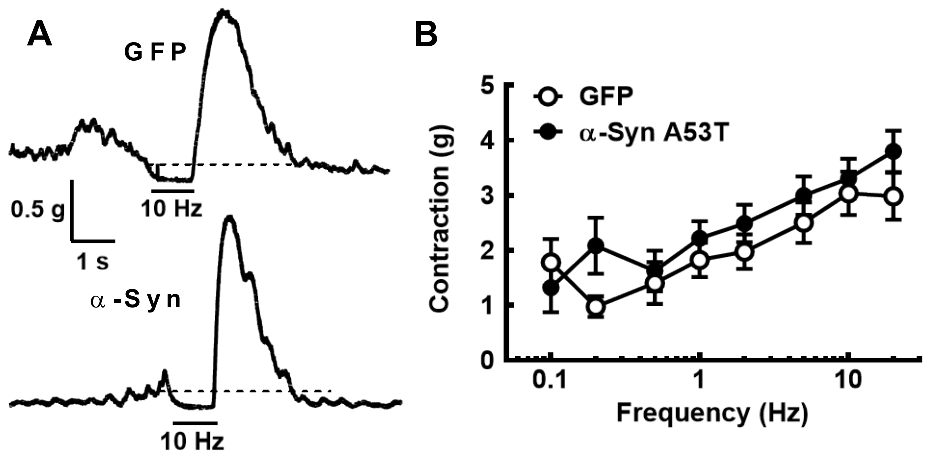

Figure 10. Neurogenic contractions evoked by transmural stimulation of the circular muscle of rat distal colon. *A. Representative recordings of neurogenic contraction of circular muscle preparations obtained from a GFP control rat (top) and an α-syn injected rat. Contractions were evoked using a 10 Hz, 1 sec train of electrical stimulation. B. Frequency response curves for contractions evoked by 10 stimuli applied at frequencies of 0.1 to 20 Hz. There were no differences in the curves obtain in tissues from GFP or α-syn injected rats (P > 0.05).*

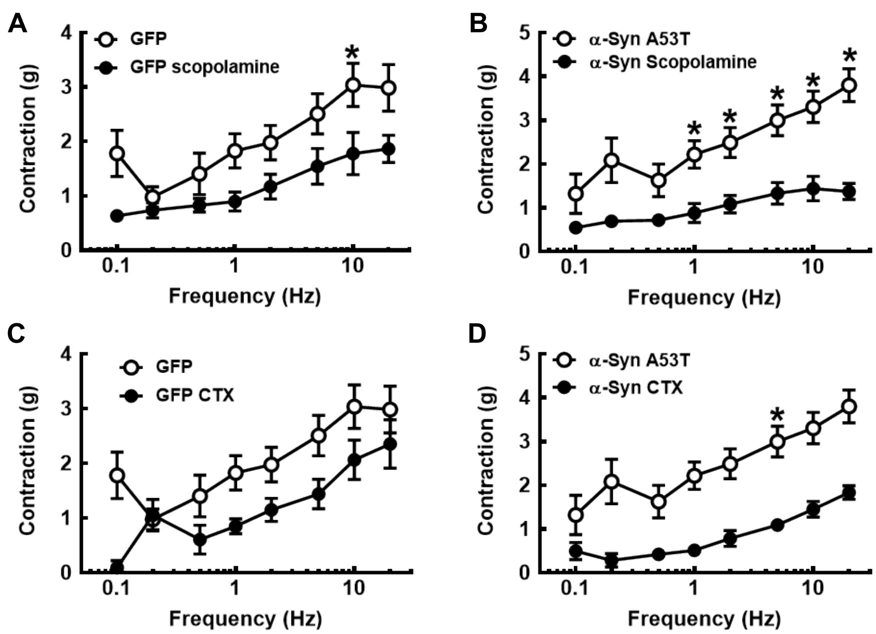

Figure 11. Frequency response curves for transmural electrical stimulation evoked contractions of rat colon circular muscle preparations in the absence and presence of scopolamine or a-conotoxin (CTX). *A. The muscarinic antagonist, scopolamine (1 uM)*

inhibited contractions in GFP injected rat colon preparations and in α-syn injected colon preparations (B). C. The N-type Ca2+ channel blocker CTX (0.1 uM) reduced the amplitude of inhibited neurogenic contractions in GFP injected colon preparations (although the reduction was not statistically significant) while CTX produced a statistically significant decrease in contraction amplitude in α-syn injected colon preparations (P<0.05).

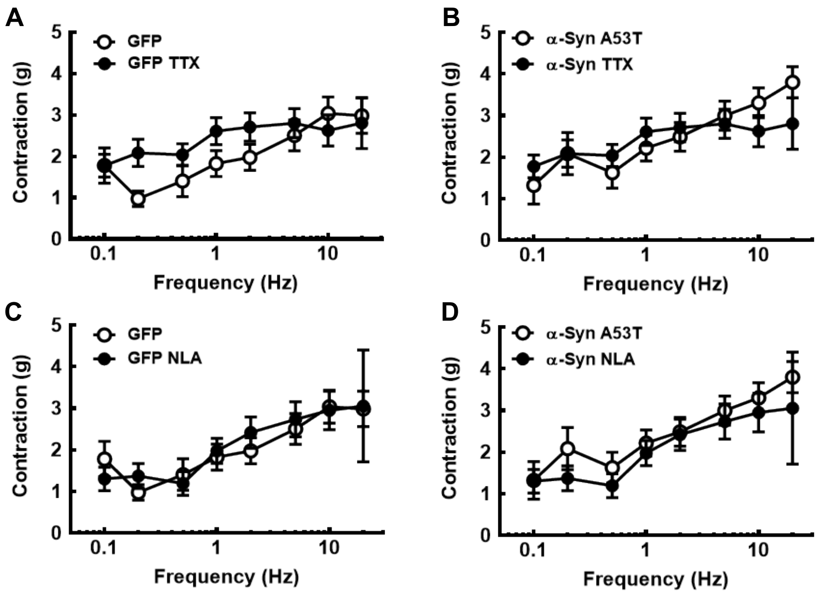

Figure 12. Frequency response curves for transmural electrical stimulation evoked contractions of rat colon circular muscle preparations in the absence and presence of tetrodotoxin (TTX) or nitro-L-arginine (NLA). *A. TTX (0.3 uM), the sodium channel blocker, did not affect contractions of GFP or (B) α-syn injected preparations (B). NLA, the nitric oxide synthase inhibitor (100 uM), did not affect electrically evoked contractions for GFP (A) or α-syn treated preparations.*

<u>Summary of activities and findings year</u>

In year 3 we finished all ex vivo analysis. Interestingly, our analysis of IJP and neurogenic contractions of the colon suggests that α-syn does not affect these measures to a great extent, despite the persistent reduction in colonic motility. One explanation for these observations is that α-syn affects a specific subset of neurons in particular. Thus in the histological outcomes we will focus on characterizing the role of phenotypically distinct cells.

Nevertheless, it is clear that ectopic α-syn overexpression in enteric neurons has an effect on gut motility and enteric neuron function. However, the finding that nitrergic signaling may be affected is not entirely in line with our overarching hypothesis. We originally posited that α-syn overexpression would interfere with vesicular neurotransmission. However, several possible explanations for this discrepancy exist. For instance, α-syn may interfere with the linkage of NO with the presynaptic space, a role that would be similar to its presumptive role in vesicular neurotransmission. On the other hand, α-syn overexpression in cholinergic interneurons may affect signaling in downstream (NO) neurons. As shown in the original proposal and previous reports- AAV readily transduces both these populations. Finally, we also need to ensure that the observed effect is not due to loss of neurons.

Finally, given an increased appreciation for the immune system in the development of neurodegenerative disorders such as PD, we also propose an alternate hypothesis: that a peripheral exposure to PD pathology (i.e. aggregated α-syn) can induce systemic inflammation that perhaps play a role in both GI function as well as initiation of central disease. To that end, and although beyond the scope of the original proposal, we have also banked blood at monthly intervals from the long-term cohort. We will analyze these for the presence of immune markers.

- **What opportunities for training and professional development has the project provided?**
  - *Nothing to Report.*
- **How were the results disseminated to communities of interest?**
  - *Nothing to Report.*
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - N/A

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*
   - **What was the impact on the development of the principal discipline(s) of the project?**
     - Our findings thus far further lend support to the hypothesis that an increased level of (pathological) α-syn in enteric neurons *per se* is sufficient to induce GI dysfunction. It is important to note that as we validated this vector-mediated approach we determined that no expression of transgene was seen either in muscle or in other neuronal populations (e.g. no virus was transported to the brain). Thus, a crucial concept that is beginning to arise from these findings is that as the field moves towards the development of therapeutics aimed at treating GI symptoms, one should focus on the PNS and not necessarily the CNS.
     - Moreover, although we have not completed the analysis of the tissue from animals receiving the original vector; it is unlikely that the low levels of ectopic α-syn that we observed in the tissue of these animals is sufficient to induce significant aggregation; *yet*, we observed a significant decline in GI function. This would argue that, at least in some instances, the role of α-syn in GI dysfunction is not related to aggregation of this protein (which is a histopathological finding in the ENS of PD patient), but rather, changes in the physiology of α-syn expressing ENS neurons.
     - Along the lines of that mentioned in the point above, our data is also providing further support to the notion that peripheral α-syn pathology can result in dysfunction of peripheral neurons, unrelated to central nervous system pathology and function.
     - We observed ENS neuronal loss concomitant with α-syn overexpression. This is an important finding, and additional analyses are underway.
   - **What was the impact on other disciplines?**
     - Although unfortunate, it was nonetheless an important observation that the specific genome originally utilized in this study failed to induce significant transgene expression. As mentioned elsewhere, we believe that the incorporation of the two promoters resulted in low stability of the genome.

We are now performing additional studies aimed at confirming this. This finding will be important to the field of gene therapy as a whole as many approaches use genomes with multiple promoters.

    - As eluded to above, we are also now interested in the role of inflammation; thus, analysis of blood from these animals may help address questions in that space as well.
    - We have donated tissue from treated animals for epigenetic analyses. Our findings indicate that enteric α-syn overexpression results in significant changes in certain pathways. The results from this work is currently under review in *Gut*.

- **What was the impact on technology transfer?**
    - Nothing to Report
- **What was the impact on society beyond science and technology?**
    - Nothing to Report

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**

  In year 3 we finalized all planned ex vivo analysis. Nevertheless, as noted above we did find potential neurodegeneration with α-syn overexpression. Thus, in the final stages of this study we plan to do additional analyses.

- **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**

  N/A

- **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**
  Report only the major publication(s) resulting from the work under this award.
  - **Journal publications.**

    Neurobiol Dis. 2018 Apr;112:106-118. doi: 10.1016/j.nbd.2018.01.008. Epub 2018 Jan 16.

    Induction of alpha-synuclein pathology in the enteric nervous system of the rat and non-human primate results in gastrointestinal dysmotility and transient CNS pathology.

    Manfredsson FP1, Luk KC2, Benskey MJ3, Gezer A4, Garcia J3, Kuhn NC3, Sandoval IM5, Patterson JR3, O'Mara A6, Yonkers R6, Kordower JH7.Federal support acknowledged

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

    Nothing to report

  - **Other publications, conference papers, and presentations.**

Epigenetic inactivation of the autophagy–lysosomal system in the Parkinson's disease appendix

Juozas Gordevicius1,2, Peipei Li2, Lee Marshall2, Bryan Killinger2,3, Sean Lang2, Elizabeth Ensink2, Nathan C. Kuhn4, Wei Cui5, Nazia Maroof6, Roberta Lauria6, Christina Rueb6, Juliane Siebourg-Polster7, Pierre Maliver7, Jared Lamp4,8, Irving Vega4,8, Fredric P. Manfredsson4, Markus Britschgi6, Viviane Labrie2,

Gut In review

Nothing to report

Technologies or techniques

Nothing to report

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

Nothing to report

- **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
- **What individuals have worked on the project?**
    - *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."*

Example:

Name:	*Fredric Manfredsson*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	ORCID 0000-0001-5802-5487
Nearest person month worked:	2
Contribution to Project:	*Dr. Manfredsson has participated in all aspects of this proposal. This includes overseeing vector generation and animal surgeries.*
Funding Support:	

Name:	*James Galligan*
Project Role:	*Co-I*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	*Dr. Galligan has assisted with animal surgeries and assisted with telemetry data analysis.*
Funding Support:	

Name:	*Xiaochun Bian*
Project Role:	*Co-I*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Bian has assisted with animal surgeries and telemeter implants.*
Funding Support:	

Name:	*Nathan Kuhn*
Project Role:	*Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4*
Contribution to Project:	*Mr. Kuhn has been involved in all aspects in this proposal. This includes assistance with, surgeries, colon transit assay, animal care, etc.*
Funding Support:	

Name:	*Mattew Benskey*
Project Role:	*Post-Doc*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Dr.Benskey (together with Dr. Manfredsson has been responsible for the execution of all aspects of this proposal thus far. Dr. Benskey assisted with colon transit assays, and participated in animal surgeries.*
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - *Nothing to Report.*
- **What other organizations were involved as partners?**
  - *Nothing to Report.*

7. **SPECIAL REPORTING REQUIREMENTS**
   - **COLLABORATIVE AWARDS:** *N/A*
   - **QUAD CHARTS:** *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*
8. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report.* ***DO NOT RENUMBER PAGES IN THE APPENDICES.***