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

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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.					
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Table of Contents

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	4
3. Accomplishments.....	4-9
4. Impact.....	9-10
5. Changes/Problems.....	10-11
6. Products.....	11
7. Participants & Other Collaborating Organizations.....	12-14
8. Special Reporting Requirements.....	14
9. Appendices.....	14

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.

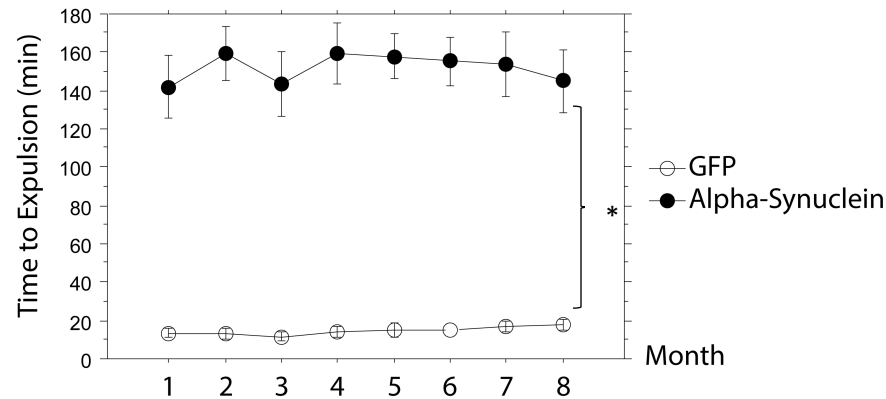
3. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.*

○ 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 are currently repeating all injections with new vector batches. This is approximately 40% complete. However, we anticipate that all animals will be injected before the end of 2017 (this is a priority in the lab in order to avoid excessive delays).

- We have performed *in vivo* colonic motility assay on all animals in the long term group.
 - We performed *ex vivo* force transduction assays and histological analysis of some animals in shorter time points. This will however be repeated in new subjects.
 - All the outcome measures described will be repeated with animals injected with new vector batches, thus at this time these measures are 0% complete.
- **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, ie. 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* Figure 2).

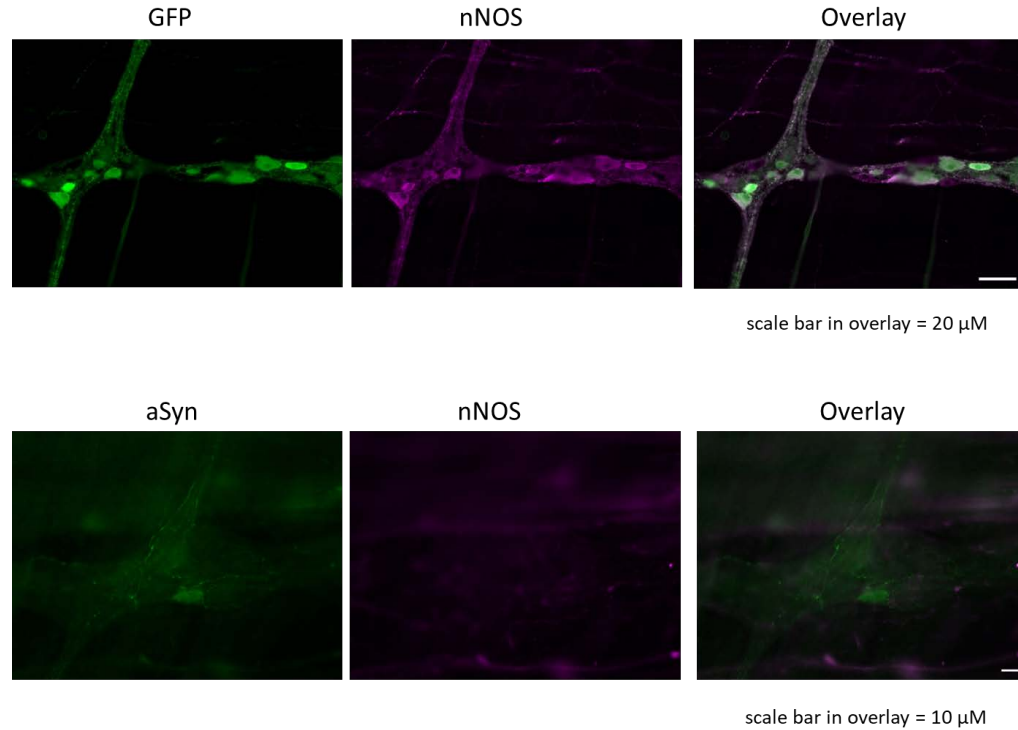


Figure 2. Initial assessment of transduction efficacy of control vector (top panels) or aSyn (lower panels). It is clear that aSyn immunoreactivity is very weak (in contrast to the GFP control transgene).

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 1×10^{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 3, this vector is producing much stronger transgene levels using the same dose.

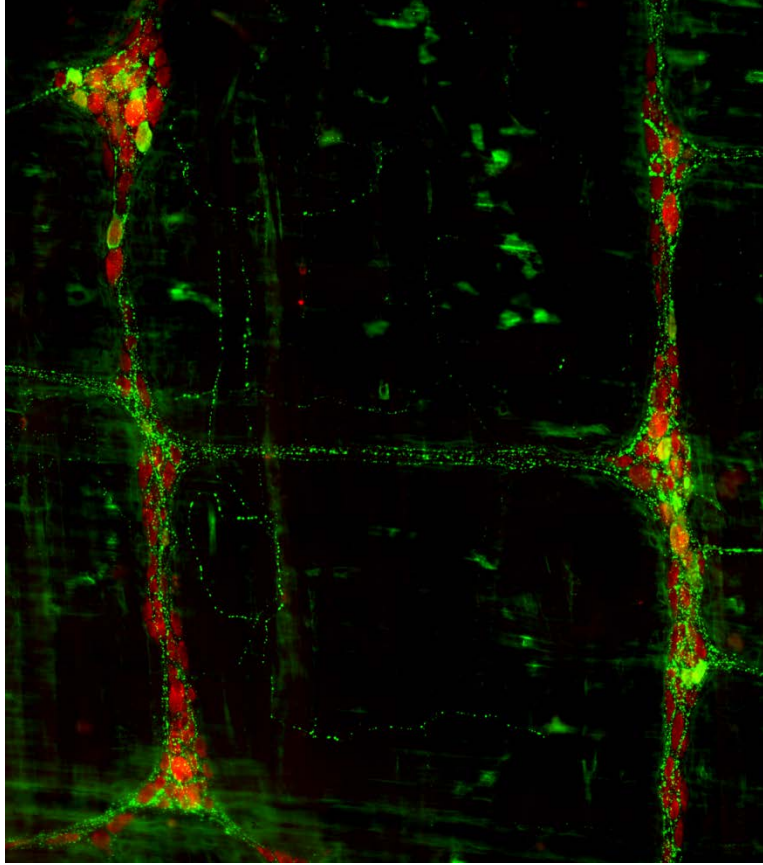


Figure 3. Transduction following delivery of rebuilt vector. The α -syn vector was delivered using the exact parameters described in the proposal and as that used in animals shown in Figure 2. Robust α -syn (green) expression can be seen throughout the neurons (HuC/D; red) and their projections.

We have also implanted telemeters in the first set of animals. At this point we have not analyzed all the telemetry data collected thus far, and the group size ($n=2/\text{treatment}$) is very small. Accordingly, the results thus far must be viewed with these limitations in mind. Nevertheless, a qualitative assessment of the data is in agreement with the bead expulsion data: namely, animals that received AAV- α -syn exhibit smaller magnitude of contractile bursts (*see* Figure 4).

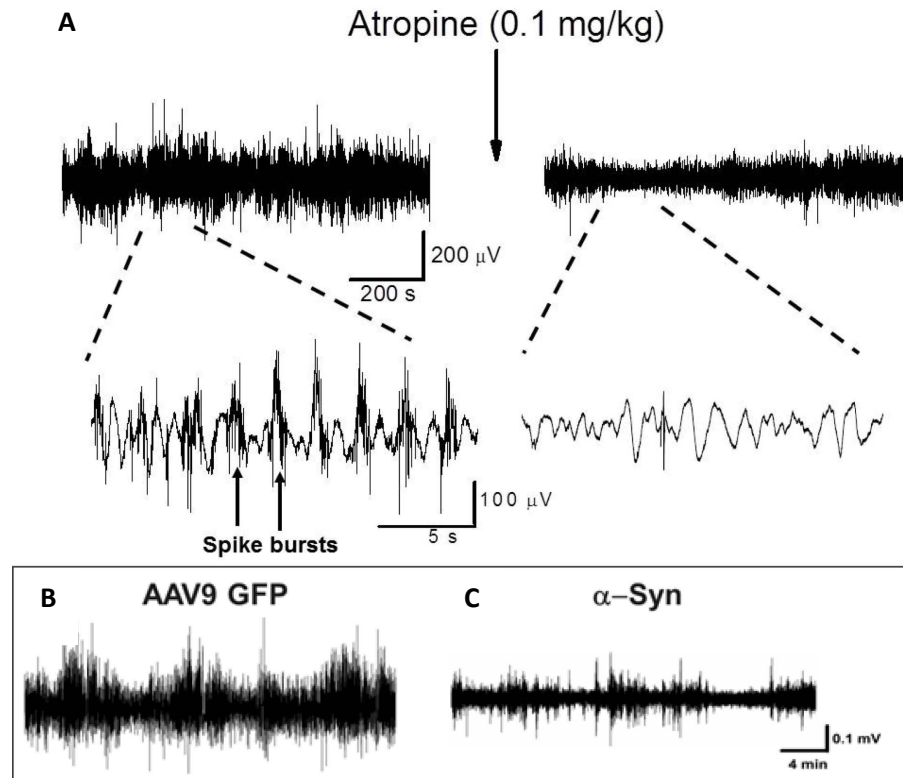


Figure 4. Radiotelemetry recordings of colon myoelectric activity in freely moving rats. **A.** Upper recordings show a 16 min recording before and after atropine treatment. Lower traces show expanded time scale recordings. Left trace shows intermittent spike bursts that are associated with contraction, right trace shows that the spike bursts are blocked by atropine. **B-C.** Recordings done 2 weeks after injection with (B) AAV-BFP2 or (C) AAV- α -syn.

Summary of activities and findings year 1

In year 1 we performed a large number of vector surgeries, generating all the subjects for the long-term analysis, and some for shorter time points. Moreover, we performed a limited amount of *ex vivo* and post-mortem analyses. Because of the issues with the vector constructs we are currently generating additional subjects to replace those that received the original vector. We are doing this in an accelerated fashion and have currently generated approximately 40% of subjects for all groups.

Although we observed a significantly lower than anticipated transduction in animals treated in year 1; the findings thus far are of still of importance: Our data shows that even low level of ectopic (presumably pathological) α -syn expression is sufficient to facilitate ENS/GI dysfunction. Although the study of these “low-dose” subjects is beyond the scope of this proposal, we will continue to follow the animals already generated, and fill out groups as needed. This will allow us to evaluate a dose-response in the final analysis.

- **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?**
 - Generate all remaining subjects (with new vector preparations)
 - Implant additional telemeters
 - Perform *in vivo* bead assay and *ex vivo* force transduction assay on all new subjects.
 - Perform histological analysis in all new subjects
 - Outside of the scope of this proposal we will also continue the analysis of animals that received the original vector. This will allow us to assess a dose-response and further strengthen the overall goals associated with this proposal.
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 were also able to detect changes in GI (colon) function in the awake animal using telemetry recordings (measuring contractions of the colon). This will become a very powerful tool moving forward as we will be able to measure physiological changes within the same subject over time.
- **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.
- **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**

As we started performing *ex vivo* outcome measures we noted that transduction (i.e. weak α -syn expression) was not as strong as anticipated, or as seen in preliminary studies (*see* Figure 2). The reason for this was identified to be some type of instability of the viral vector genome (more than likely promoter recombination). We redesigned the viral vector and tested this in pilot animals. As can be seen in Figure. 3, transduction with the remade vector is much stronger and more closely resembles that seen in preliminary studies, and described in the proposal. Nevertheless, this means that all animals generated in the first half of the current reporting year cannot be included in the study as proposed. Although these animals exhibited some gastric dysmotility, initial assessments suggest that this degree of α -syn expression is not sufficient to induce aggregation. We have begun to remake all these subjects. We do, however, expect that in order to complete the study as outlined in the original proposal that we will have to request a no-cost extension at the end of year 2. Importantly, in order to not let animals already generated to go to waste, we will perform biochemical and histological analyses akin to those described in the proposal (using discretionary funds and

resources not originating from this proposal). This will allow us to determine the role of α -syn dosage in ENS dysfunction.

- **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.**

- Title: 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

Fredric Manfredsson, PhD; Kelvin C Luk; Matthew Benskey; Aysegul Guezer; Joanna Garcia; Nathan Kuhn; Ivette Sandoval; Joseph Patterson; Alana O'Mara; Reid Yonkers; Jeffrey Kordower

Neurobiology of disease-In review

Federal support acknowledged

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

Nothing to report

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

Nothing to report

Website(s) or other Internet site(s)

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

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

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

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

Name:	<i>Matthew Benskey</i>
Project Role:	<i>Post-Doc</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>6</i>
Contribution to Project:	<i>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.</i>
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.***

- Appendix 1: Article currently under review in Neurobiology of disease

Manuscript Number: NBD-17-700

Title: 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

Article Type: Research paper

Keywords: Alpha-synuclein; Enteric Nervous System; Parkinson's disease; Enteric dysfunction; Prion Spread

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First Author: Fredric Manfredsson, PhD

Order of Authors: Fredric Manfredsson, PhD; Kelvin C Luk; Matthew Benskey; Aysegul Guezer; Joanna Garcia; Nathan Kuhn; Ivette Sandoval; Joseph Patterson; Alana O'Mara; Reid Yonkers; Jeffrey Kordower

Abstract: Alpha-Synuclein (α -syn) is by far the most highly vetted pathogenic and therapeutic target in Parkinson's disease. Aggregated α -syn is present in sporadic Parkinson's disease, both in the central nervous system (CNS) and peripheral nervous system (PNS). The enteric division of the PNS is of particular interest because 1) gastric dysfunction is a key clinical manifestation of Parkinson's disease, and 2) Lewy pathology in myenteric and submucosal neurons of the enteric nervous system (ENS) has been referred to as stage zero in the Braak pathological staging of Parkinson's disease. The presence of Lewy pathology in the ENS and the fact that patients often experience enteric dysfunction before the onset of motor symptoms has led to the hypothesis that α -syn pathology starts in the periphery, after which it spreads to the CNS via interconnected neural pathways. Here we sought to directly test this hypothesis in rodents and non-human primates (NHP) using two distinct models of α -syn pathology: the α -syn viral overexpression model and the preformed fibril (PFF) model. Subjects (rat and NHP) received targeted enteric injections of PFFs or adeno-associated virus overexpressing the Parkinson's disease associated A53T α -syn mutant. Rats were evaluated for colonic motility monthly and sacrificed at 1, 6, or 12 months, whereas NHPs were sacrificed 12 months following inoculation, after which the time course and spread of pathology was examined in all animals. Rats exhibited a transient GI phenotype that resolved after four months. Minor α -syn pathology was observed in the brainstem (dorsal motor nucleus of the vagus and locus coeruleus) 1 month after PFF injections; however, no pathology was observed at later time points (nor in saline or monomer treated animals). Similarly, a histopathological analysis of the NHP brains revealed no pathology despite the presence of robust α -syn pathology throughout the ENS which persisted for the entirety of the study (12 months). Our study shows that induction of α -syn pathology in the ENS is sufficient to induce GI dysfunction. Moreover, our data suggest that sustained spread of α -syn pathology from the periphery to the CNS and subsequent propagation is a rare event, and that the presence

of enteric α -syn pathology and dysfunction may represent an epiphenomenon.

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October 9, 2017

Dr. Timothy Greenamyre

Editor

Neurobiology of Disease

RE: Submission of Research Article to Neurobiology of Disease

"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"

Dear Dr. Greenamyre,

We are very pleased to submit our manuscript demonstrating the long-term consequences following the delivery of pathologic alpha-synuclein (α -syn; either in the form of pre-formed fibrils or via overexpression) to the enteric nervous system of the rat and non-human primate. Our findings suggest that enteric α -syn pathology, on its own, is not capable of sustainable spread and propagation throughout the CNS. However, we do find that enteric α -syn pathology is sufficient to induce colonic dysmotility. It is important to point out that our findings do not contradict other manuscripts published elsewhere as we do observe the transient presence of synuclein pathology in the brainstem. Moreover, although our findings in one sense are negative, this is still incredibly important information for the field. This is a very timely manuscript which will be of great interest to a broad range of Parkinson's disease/ α -syn researchers and clinicians, and it touches upon an extremely important concept that is applicable to all synucleinopathies: is peripheral synuclein pathology the origin of CNS disease? Thus, this manuscript is well suited for a broad range of Neurobiology of Disease readership.

All authors have reviewed and approve of the contents of this manuscript. This work is not under consideration elsewhere. The authors have no conflicts of interest to declare.

We suggest the following reviewers because of their significant experience with α -syn biology, neuropathology, and neurogastroenterology:

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Thank you for this opportunity to submit our manuscript.

Sincerely,



Fredric P. Manfredsson, Ph.D.

Assistant Professor

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

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Running Title:

Enteric α -syn pathology causes transient CNS pathology

Abstract

Alpha-Synuclein (α -syn) is by far the most highly vetted pathogenic and therapeutic target in Parkinson's disease. Aggregated α -syn is present in sporadic Parkinson's disease, both in the central nervous system (CNS) and peripheral nervous system (PNS). The enteric division of the PNS is of particular interest because 1) gastric dysfunction is a key clinical manifestation of Parkinson's disease, and 2) Lewy pathology in myenteric and submucosal neurons of the enteric nervous system (ENS) has been referred to as stage zero in the Braak pathological staging of Parkinson's disease. The presence of Lewy pathology in the ENS and the fact that patients often experience enteric dysfunction before the onset of motor symptoms has led to the hypothesis that α -syn pathology starts in the periphery, after which it spreads to the CNS via interconnected neural pathways. Here we sought to directly test this hypothesis in rodents and non-human primates (NHP) using two distinct models of α -syn pathology: the α -syn viral overexpression model and the preformed fibril (PFF) model. Subjects (rat and NHP) received targeted enteric injections of PFFs or adeno-associated virus overexpressing the Parkinson's disease associated A53T α -syn mutant. Rats were evaluated for colonic motility monthly and sacrificed at 1, 6, or 12 months, whereas NHPs were sacrificed 12 months following inoculation, after which the time course and spread of pathology was examined in all animals. Rats exhibited a transient GI phenotype that resolved after four months. Minor α -syn pathology was observed in the brainstem (dorsal motor nucleus of the vagus and locus coeruleus) 1 month after PFF injections; however, no pathology was observed at later time points (nor in saline or monomer treated animals). Similarly, a histopathological analysis of the NHP brains revealed no pathology despite the presence of robust α -syn pathology throughout the ENS which persisted for the entirety of the study (12 months). Our study shows that induction of α -syn pathology in the ENS is sufficient to

induce GI dysfunction. Moreover, our data suggest that sustained spread of α -syn pathology from the periphery to the CNS and subsequent propagation is a rare event, and that the presence of enteric α -syn pathology and dysfunction may represent an epiphenomenon.

Key words: Alpha-synuclein; Enteric Nervous System; Parkinson's disease; Enteric dysfunction; Prion Spread

Introduction

Alpha-Synuclein (α -syn) is currently the most vetted participant in Parkinson's disease pathology. Mutations in, and multiplications of, the gene encoding α -syn are associated with familial forms of the disease, and polymorphisms in the gene confer increased risk in developing Parkinson's disease (Polymeropoulos *et al.*, 1997; Singleton *et al.*, 2003; Ibanez *et al.*, 2004; Simon-Sanchez *et al.*, 2009). In both familial and sporadic Parkinson's disease, α -syn is a major component of Lewy bodies and Lewy neurites, the pathological hallmark of Parkinson's disease (Spillantini *et al.*, 1997). Finally, ectopic overexpression of α -syn in rodents and non-human primates results in a dose-dependent and stereotypic pattern of neurodegeneration that is relatively specific to nigrostriatal neurons (Kirik *et al.*, 2002; Eslamboli *et al.*, 2007; Gombash *et al.*, 2013). Although the molecular etiology underlying α -syn-mediated neurotoxicity is yet to be defined, it is clear that oligomerization and fibrillization of the protein play a central role in the pathology. As such, it has been argued that α -syn aggregates, or intermediate forms of aggregates, are directly toxic to cellular organelles and/or processes. The alternate viewpoint contends that the role of α -syn in neurodegeneration is that of a toxic loss of function as a result of sequestration of the monomeric (and presumptive functional) form of α -syn in to insoluble aggregates (Benskey *et al.*, 2016a; Dettmer *et al.*, 2016).

Regardless of the modality by which aggregates cause neurotoxicity, recent advances in the field suggest that pathological, aggregated, forms of α -syn can spread throughout the nervous system via cycles of transmission, templating, and aggregation of α -syn in recipient cells, in a process that propagates along the neuraxis (Volpicelli-Daley *et al.*, 2011; Luk *et al.*, 2012a; Paumier *et al.*, 2015). The first indication that α -syn pathology can spread was observed in patients receiving fetal mesencephalic neuronal transplants (Kordower *et al.*, 2008; Li *et al.*,

2008; Chu and Kordower, 2010; Li *et al.*, 2010; Li *et al.*, 2016; Kordower *et al.*, 2017). Post-mortem histological analyses revealed that a portion of the transplanted (presumably healthy) tissue contained Lewy pathology, with the degree of Lewy pathology being time-dependent. These data suggested that α -syn pathology had spread from the host to the graft; a finding supported by preclinical experiments (Hansen *et al.*, 2011; Kordower *et al.*, 2011). This finding was followed up experimentally with research suggesting that recombinant α -syn pre-formed α -syn fibrils (PFF) can transmit from cell to cell. Stereotaxic injections of PFFs in to various regions of the central nervous system (CNS) results in the aggregation of endogenous α -syn in a wide range of anatomically distinct loci throughout the brain, with concomitant neuronal loss in areas such as the substantia nigra (Luk *et al.*, 2012a; Paumier *et al.*, 2015). Although unequivocal evidence of actual *in vivo* neuron-to-neuron transmission is still lacking, interneuronal transmission has been observed in microfluidic devices between cultured neurons *in vitro* (Volpicelli-Daley *et al.*, 2011; Brahic *et al.*, 2016) and these processes are dependent upon the binding of pathological α -syn to the lymphocyte-activation gene 3 (LAG3) (Mao *et al.*, 2016) and transmembrane protein 9 superfamily member 2 (TM9SF2; preliminary data from the laboratory of Mark Cookson (Wadman, 2016)) receptors.

The potential propagation of α -syn pathology throughout the CNS holds great explanatory power as it applies to the varying staging schemes of Parkinson's disease progression (Braak *et al.*, 2003a). However, it has become increasingly recognized that α -syn pathology is not limited to the CNS; rather, α -syn (i.e. Lewy) pathology has been observed throughout the peripheral nervous system (PNS) as well. α -syn pathology has been observed in Parkinson's disease patients in organs such as skin (Ikemura *et al.*, 2008), heart (Iwanaga *et al.*, 1999), and the enteric nervous system (ENS) (Wakabayashi *et al.*, 1988; Wakabayashi *et al.*,

1990; Braak *et al.*, 2006; Beach *et al.*, 2010). The ENS is of particular interest, because gastric dysfunction is a key component of Parkinson's disease symptomology (Edwards *et al.*, 1991; Pfeiffer, 2011; Park *et al.*, 2015). Moreover, symptoms such as constipation are present years prior to an established diagnosis (Ross *et al.*, 2012; Sung *et al.*, 2014; Chen *et al.*, 2015). Indeed, Lewy pathology in myenteric and submucosal neurons of the ENS is sometimes referred to as stage zero in the classic Braak staging of Parkinson's disease (Braak *et al.*, 2003b). Although a direct correlation between enteric Lewy pathology and gastrointestinal (GI) dysfunction in Parkinson's disease is yet to be made, a GI phenotype has been observed in transgenic animals overexpressing α -syn (Wang *et al.*, 2008; Kuo *et al.*, 2010; Hallett *et al.*, 2012; Farrell *et al.*, 2014).

The presence of Lewy pathology in the ENS and the fact that patients experience ENS dysfunction before the onset of motor symptoms has led to the hypothesis that α -syn pathology originates in peripheral neurons, specifically within neurons of the ENS, from where it spreads to the CNS. Congruent with this idea is the fact that enteric α -syn pathology has been detected in patients *prior* to a Parkinson's disease diagnosis (Shannon *et al.*, 2012a), but *see* Beach *et al.*, 2016 (Beach *et al.*, 2016). Moreover, the presumptive route of transmission is thought to be via vagal innervation, due to the fact that brainstem nuclei such as the dorsal motor nucleus of the vagus display Lewy pathology early in the course of the disease. Further, direct delivery of adeno-associated virus (AAV) overexpressing human α -syn (h α -syn) into the vagus nerve results in spread of exogenous α -syn extending rostrally throughout the brainstem (Ulusoy *et al.*, 2013). Moreover, injection of human Parkinson's disease brain lysate in to the wall of the intestine of rodents results in the transport of exogenous α -syn in the vagus nerve to the dorsal motor nucleus

of the vagus (Holmqvist *et al.*, 2014). Thus, ample scientific evidence exists in support of the “synuclein-spreading” hypothesis of Parkinson’s disease.

Herein we took a multi-modal approach in order to determine whether α -syn pathology can be initiated in the periphery (i.e. ENS) and spread to the CNS over time. We injected PFFs directly in to the myenteric layer of either the rat colon, or the non-human primate (NHP) colon and stomach. In addition, a separate cohort of non-human primates received localized enteric injections with AAV overexpressing the Parkinson’s disease associated A53T α -syn mutant form. Rodents were monitored for colonic dysmotility for up to one year and sacrificed at intermediate time points in order to monitor the progression of α -syn pathology. Similarly, NHPs were sacrificed one year after α -syn or vector delivery and assessed for CNS and ENS pathology.

Materials and Methods

Vector Production

Recombinant AAV (rAAV) genomes were the same as those used in previous studies investigating α -syn overexpression and neurodegeneration in the CNS (Gombash *et al.*, 2013): Coding sequences for human α -syn (h α -syn) A53T or GFP were under the control of the chicken beta-actin (CBA)/cytomegalovirus (CMV) promoter hybrid. Genomes were packaged in to AAV5 capsids as previously described (Benskey *et al.*, 2016b). HEK 293T cells were transfected with plasmids encoding the respective genomes together with plasmids encoding helper functions. 72 hours following transfection cells were harvested and media was collected and concentrated using tangential flow filtration. Viral particles were purified using an iodixanol

gradient followed by column chromatography. Titers were determined via dot-blot (Zolotukhin *et al.*, 1999) and normalized to 1×10^{13} vector genomes/ml (vg/ml) using balanced salt solution (Alcon, Fort Worth, TX).

Generation of PFFs

Production of recombinant mouse α -syn (for rat studies) and human α -syn (for NHP studies) and *in vitro* fibril assembly was performed as described (Luk *et al.*, 2012a; Volpicelli-Daley *et al.*, 2014). Mouse PFFs were identical to those previously used within the mouse and rat CNS (Luk *et al.*, 2012a; Paumier *et al.*, 2015). Prior to the surgeries PFFs were thawed, diluted in dPBS, and sonicated at room temperature using an ultrasonic homogenizer (300VT; Biologics, Inc., Manassas, VA; pulser at 20%, power at 30%, 60 pulses at 1 s each). Recombinant α -syn monomer was thawed on ice, diluted in ice-cold sterile saline, and kept on ice throughout the surgical session. Sonicated fibrils were visualized using electron microscopy (EM). Formvar/carbon coated copper grids (EMS DIASUM: FCF300-Cu) were washed twice with ddH₂O and floated for 1 minute on a 10 μ l drop of sonicated α -syn fibrils diluted 1:20 with DPBS. Grids were stained for 1 minute on a drop of 2% uranyl acetate aqueous solution; excess uranyl acetate was wicked away with filter paper, and grids were allowed to dry before imaging. Grids were imaged on a JEOL JEM-1400 transmission electron microscope. The length of over 500 fibrils per sample was measured and the data was analyzed and graphed in GraphPad Prism 7 and the average fibril length from each sample was determined. A majority of the fibril species was at a length considered to be ideal for seeding (mean = $55.9 \text{ nm} \pm 1.1 \text{ nm}$ for mouse fibrils (n=641) and $75.8 \text{ nm} \pm 2.2 \text{ nm}$ for human fibrils (n=520) (Tarutani *et al.*, 2016; Abdelmotilib *et al.*, 2017); (Sup. Fig. 1).

Surgeries-Rat

Young adult (220g) Sprague-Dawley male rats were utilized in accordance with the Michigan State University Institutional Animal Care & Use Committee (AUF 08/12-150-00) guidelines. Injections directly targeted to the enteric neurons of the descending colon were performed as previously described (Benskey *et al.*, 2015b; Benskey and Manfredsson, 2016). Briefly, rats were anesthetized using isoflurane and a full laparotomy was performed in order to isolate and expose the descending colon. The proximal border of the area to be injected was demarcated with a tattoo, and 6 x 5 μ l injections of PFFs (2 μ g/ μ l; n = 15, recombinant monomeric α -syn (2 μ g/ μ l; n = 15), or a saline vehicle control (n = 14) were administered distal to the tattoo. Injections were performed with an automated micropump at 1 μ l/s. Following the injection the needle was left in place for an additional 10-20 seconds to allow the injection pressure to dissipate and prevent reflux.

Fecal Output Assay

Animals in the longest survival group (12 months) were evaluated monthly for changes in colonic motility (Devries *et al.*, 2010). Rats were food deprived for 24 hours prior to the start of the experiment. Rats were housed singly for a 12-hour period, during which time fecal pellets were collected, counted, and weighed every 3 hours. Following the final collection, the total fecal matter collected from each animal was dried overnight at 60 °C to remove water and weighed a final time in order to calculate water content of feces.

NHP surgeries

All non-human primate work was conducted in accordance with Rush University Medical Center Institutional Animal Care & Use Committee. NHPs (*Macaca fascicularis*) were tranquilized with ketamine (10 mg/kg) and then anesthetized with isoflurane (1-2%). Under sterile conditions, a midline incision was made and the stomach and intestines were exteriorized. Injections of PFF's (n = 4), monomer (n = 2), AAV2/5-alpha syn A53T (n = 4), AAV2/5-GFP (n = 3), or saline vehicle (n = 2) were made into the colon (9 injections) and stomach (1 injection). All injections were made in 10ul volumes (2µg/µl). Non-absorbable sutures were placed at the rostral and caudal margins of the injection sites to facilitate identification of the injection sites post-sacrifice. The organs were replaced into their original positions and the incision was closed in anatomical layers. All monkeys recovered uneventfully.

Tissue Collection and Processing-Rat

1, 6, or 12 months following injections, rats were euthanized with a lethal injection of sodium pentobarbital and transcardially perfused with Tyrode's solution. Whole mount colon tissue was collected and prepared as previously described (Benskey *et al.*, 2015b), and small portions of the injected colon was dissected by separating the myenteric and submucosal layers. The brains were harvested and post-fixed in 4% paraformaldehyde (in TBS) for 72 hours followed by cryoprotection in 30% sucrose. Brains were sectioned coronally in to 40 µm sections using a sliding stage microtome.

Tissue Collection and Processing-NHP

Twelve months post-surgery, all monkeys were anesthetized with pentobarbital (25 mg/kg, iv) and perfused with warm followed by ice cold saline. The stomach and intestines were removed and immersion fixed in 4% paraformaldehyde. The brain was removed and placed in

ice cold saline, slabbed on a calibrated brain slice apparatus and immersion fixed in 4% paraformaldehyde.

Immunohistochemistry and Proteinase K treatment

Monkey colon and stomach samples were collected near the injection site (demarcated by a suture in the mesentery). The collected monkey tissue was embedded in paraffin and sectioned in to 8µm sections. Prior to immunohistochemical detection, paraffin embedded tissue was dewaxed and processed for antigen retrieval and removal of connective tissue (Fried and Gulbransen, 2015). Briefly, sections were rinsed in H₂O and incubated in 10mM sodium citrate buffer (pH 6.0) for 30 minutes at 80 °C, then cooled to room temperature for 30 minutes. Tissue was then rinsed in H₂O and incubated in 150U/ml (0.7 mg/ml) collagenase (Life Technologies, Grand Island, NY) and 1U/ml (0.6 mg/ml) dispase II (Sigma, St. Louis, MO) in DMEM for 15 minutes.

All immunohistochemical detection in rats and monkey was performed as follows for colon and brain: sections were washed in TBS containing 0.5% Triton-X 100, incubated in 3% peroxide solution and blocked in 10% normal goat serum. Neurons within enteric ganglia were identified using the pan-neuronal marker HuC/D (1:2000; Invitrogen/Life Technologies, Grand Island, NY). Sections were also probed for α -synuclein using both a pan α -synuclein antibody (1:1000; BDbioscience, San Jose, CA) and an antibody that recognizes α -synuclein phosphorylated at serine 129 (1:10000) (Waxman and Giasson, 2008; Volpicelli-Daley *et al.*, 2011). Secondary antibodies used were Goat anti-mouse IgG2a Alexa Fluor 488(1:500; Invitrogen/Life Technologies, Grand Island, NY), Goat anti-mouse IgG1 Alexa Fluor 488(1:500; Invitrogen), Goat anti-mouse IgG2b Alexa Fluor 594(1:500; Invitrogen). In rAAV-GFP treated

animals Alexa Fluor 350 was used in lieu of 488 (however, native GFP fluorescence was quenched due to the tissue processing). Sections were coverslipped using Vectorshield hard set mounting medium (VectorLabs, Burlingame, Ca).

Proteinase K treatment was performed on a subset of rat tissue prior to immunohistochemistry. Collected tissue was washed in PBS and treated in 1mg/ml Proteinase K (Fungal, Invitrogen #25530015) in TE buffer (1M Tris, 0.5M EDTA at pH 7.5) at 25°C for 10 minutes.

Sections were imaged on a Nikon eclipse 90i fluorescence microscope connected to a Q-imaging fast 1394 camera (fluorescence microscopy) or a Nikon D-1 camera (brightfield microscopy). Confocal images were captured using a Nikon Ti Eclipse microscope and images were processed using the NIS elements software. Figures were assembled using Canvas 7SE (ACD Systems, Canada).

Manual Cell Counting of DMN neurons

To quantify the number of neurons in the DMN, brainstem tissue was processed for immunohistochemical detection (as described above) of the pan-neuronal marker HuC/D. High magnification images of the DMN were obtained bilaterally, at the level of the central canal, from 3-4 adjacent sections per animal. The Image J cell counter plugin was used to manually count the number of HuC/D positive neurons within the anatomical boundaries of the DMN. The mean number of HuC/D+ DMN neurons per section was averaged over all sections within a single animal and presented as HuC/D+ neurons per section.

Statistical analysis

Colonic motility assays were performed by an experimenter blind to all experimental conditions. All statistical tests were performed using Statview and graphed using GraphPad Prism software (Version 7, GraphPad, La Jolla, CA). One-way analysis of variance (ANOVA) was used to detect differences in one independent variable between treatment groups. When appropriate, *post-hoc* comparisons were performed using the Bonferroni method. Statistical significance was set at $p \leq 0.05$.

Results

Enteric Inoculations of PFFs result in a transient reduction in colonic motility in the rat

Enteric dysfunction is a key component of Parkinson's disease and here we wanted to determine whether pathological α -syn located solely in the ENS is sufficient to alter colonic motility, and whether colonic dysmotility would progress over time. Rats received injections to the descending colon with PFFs, monomeric α -syn, or saline, and colonic motility was assessed monthly for one year using total fecal pellet output and fecal water content as an indirect measure of colonic motility (Raffa *et al.*, 1987). Both treatment with monomeric α -syn and PFFs resulted in a significant reduction in fecal water content one month after the inoculations as compared to saline treated animals at the same time point (Fig. 1A). Moreover, PFF treated rats also exhibited decreased fecal output; however, this was only significant 3 months following the injections (Fig. 1B). The effect of α -syn on colonic motility was transient, however, and no impact of treatment on colonic motility was seen with either treatment for the remainder of the study (1 year).

Enteric Inoculations of PFF Results in Persistent ENS α -syn Pathology in the Rat

We then wanted to evaluate the histopathological consequences following enteric α -syn delivery, and to assess whether the normalization of colonic motility was due to clearance of pathological α -syn from enteric neurons over time. A subset of rats were sacrificed 1, 6 or 12 months following the enteric inoculations, after which colonic whole mount preparations containing myenteric neurons were assessed for α -syn immunoreactivity. Rats inoculated with PFF exhibited a significant degree of punctate α -syn immunoreactivity (i.e. aggregated α -syn) within myenteric ganglia as well as throughout axon tracts radiating from these ganglia (Fig. 1C). As α -syn is typically presynaptically enriched (Iwai *et al.*, 1995; Benskey *et al.*, 2016a) it is likely that many of these distal aggregates coincide with myenteric varicosities. Roughly, the same degree and distribution of α -syn immunoreactivity was seen in myenteric ganglia of subjects sacrificed 6 months after the inoculation (Fig. 1D). However, less (aggregated) α -syn immunoreactivity was seen in the processes of PFF injected animals 12 months after the inoculation (Fig. 1E). Monomer injected animals also exhibited pronounced punctate α -syn immunoreactivity at 1, 6, and 12 months following the injection (Fig. 1F-H), which followed the same temporal progression to that seen in PFF animals. To confirm that the punctate α -syn immunoreactivity indeed represented insoluble inclusions a subset of whole mount preparations were processed with Proteinase K and thereafter processed for α -syn immunohistochemistry. A significant degree of Proteinase K resistant α -syn was observed in both PFF (Fig. 2 A-C) and monomer (Fig. 2 D-F) treated animals and these inclusions persisted for 12 months. Very little α -syn immunoreactivity was observed in saline injected animals at any time point examined (Fig. 1 I; Fig 2 G).

Enteric Inoculations of PFF Results in Transient Brainstem α -syn Pathology in the Rat

The presence of enteric (pathological) α -syn is observed in most Parkinson's disease patients (Wakabayashi *et al.*, 1990; Braak *et al.*, 2006) and is thought to arise prior to the onset of motor symptoms (Shannon *et al.*, 2012b). Accordingly, it has been postulated that certain forms of α -syn (e.g. fibrils) can spread from the ENS to the central nervous system where propagation occurs and pathology ensues. Thus, a key aim in this study was to assess whether enteric α -syn pathology would spread to the CNS. Our PFF injections were targeted to the descending colon where approximately 20% of ganglia are innervated by the vagus nerve (Berthoud *et al.*, 1991; Altschuler *et al.*, 1993). In order to determine whether pathological α -syn could spread from the ENS to the brainstem and beyond, a subset of rats were sacrificed 1, 6 or 12 months following the enteric inoculations with α -syn and evaluated for CNS pathology as measured by the detection of α -syn phosphorylated at serine 129 (S129); a modification commonly associated with pathological, Lewy body-associated, α -syn (Hasegawa *et al.*, 2002). S129 phosphorylated α -syn was consistently observed in PFF treated subjects sacrificed one month after the enteric injection in a small number of neurons in the dorsal motor nucleus of the vagus (DMN; Fig 3 C). Moreover, we observed the presence of phosphorylated α -syn in a few cells in the locus coeruleus (Fig 3 J) at the same time point. It is worth noting that the degree of α -syn pathology was minor, and the extent of pathology did not resemble that seen with intracerebral administration of PFF (e.g. (Paumier *et al.*, 2015); *see* Fig. 2 K for an example of nigral pathology following striatal administration). No pathology was observed in monomer or saline treated animals (Fig 3 A, B. Importantly, this pathology was not observed at later time points (Fig 3 D-I). The lack of abundant α -syn pathology at the 1-month time point, and the loss of α -syn pathology over time, was also consistent with the absence of overt cell loss in the DMN. Quantification of HuC/D+ neurons within the DMN did not reveal any change in the number of

neurons within saline or PFF treated animals at any time point examined (Fig. 3 L-N). Moreover, no neuronal loss was observed in the substantia nigra of PFF or monomer treated animals (data not shown).

Enteric Inoculations of PFF Does Not Result in Spinal Cord α -syn Pathology in the Rat

In addition to vagal innervation (Berthoud *et al.*, 1991; Altschuler *et al.*, 1993), the descending colon is also innervated by the lumbar splanchnic nerve. Accordingly, we wanted to determine whether α -syn PFFs were internalized by axon terminals originating within the intermediolateral nucleus of the spinal cord. Lumbar segments 1 and 2 of the rat spinal cord (sacrificed 1 month post injection) were processed for S129 immunoreactivity. However, no S129 immunoreactivity was seen in any sections (Sup. Fig. 2).

Enteric Overexpression of α -syn in Non-Human Primates Results in Neuronal α -syn Pathology

Viral vector-mediated overexpression of α -syn is a popular means to model nigrostriatal neurodegeneration, wherein long-term overexpression results in aggregate formation, altered neuronal function, and ultimately neurodegeneration (Kirik *et al.*, 2002; Lundblad *et al.*, 2012; Gombash *et al.*, 2013). Similarly, herein we utilized targeted delivery of rAAV directly to the ENS of the NHP stomach and colon (Benskey *et al.*, 2015b; Benskey and Manfredsson, 2016) in order to overexpress the A53T disease mutant form of α -syn specifically in enteric neurons without the confound of transgene expression in other tissues. Importantly, this direct delivery method does not result in detectable vector genomes in the brainstem (as measured by qRT-PCR) (Benskey *et al.*, 2015b). Titer matched rAAV expressing a GFP transgene was used as a control. One year following the vector delivery there was a clear presence of aggregated α -syn in enteric

neurons throughout the ganglia of both the colon (Fig. 4 P-T) and stomach (Fig. 5 P-T) as indicated by the presence of S129 phosphorylated α -syn. No S129+ α -syn was detected in rAAV-GFP treated subjects (Fig. 4 U-Z; Fig. 5 U-Z)).

Immunofluorescent imaging of enteric tissue is inherently difficult due to the strong background fluorescence of the tissue (Smith, 2011). Consequently, additional tissue was processed for brightfield imaging using immunohistochemical development in order to confirm the findings. In congruence with the fluorescent imaging, A53T overexpression correlated with significant α -syn S129 immunoreactivity throughout the treated ENS (Sup. Fig. 3 A,B). Similarly, evaluating the immunoreactivity of endogenous α -syn showed that a portion of α -syn appeared to be associated with aggregates with a punctate staining pattern observed in many cells (Sup. Fig. 3 C,D, Sup. Fig. 4 M-P, Sup. Fig. 5 M-P). However, it was also clear that a detectable degree of diffuse α -syn was present in the soma of neurons of the stomach and colon in subjects treated with AAV-A53T (e.g. Sup. Fig. 4 N-P); perhaps reflecting endogenous expression of α -syn. Interestingly, both the fluorescent and the brightfield analysis of Ser129 immunoreactivity in A53T expressing subjects demonstrated that a significant degree of phosphorylated α -syn was diffusely distributed throughout the soma and not limited to dense aggregate structures (e.g. Fig. 4 Q-S).

Confirmation of Vector Transduction

Studies using rAAV in the CNS have demonstrated stable expression for decades following the delivery (Marks *et al.*, 2016). However, such data for transduction of enteric neurons does not exist and up to this point we have only analyzed expression one month following vector delivery (Benskey *et al.*, 2015b). Accordingly, vector transduction was

confirmed by immunohistochemistry for GFP (control subjects). Numerous transgene positive neurons were observed in all ganglia surveyed (Sup. Fig. 6). Moreover, because of the degree of endogenous expression levels of α -syn it was not possible to detect the A53T transgene *per se*. Rather, we utilized PCR to confirm transduction (i.e. the presence of viral genomes) in all vector treated tissue (data not shown).

A Single Inoculation of α -syn PFFs or Monomers into the NHP ENS Result in Persistent Enteric Pathology

As with α -syn overexpression, the inoculation of either monomeric recombinant mouse α -syn or PFFs correlated with the presence of aggregated (S129+ α -syn) in neurons throughout enteric ganglia of injected portions of the colon (Fig. 4) and the stomach (Fig. 5) 12 months following the inoculations. Aggregated α -syn was not observed in saline treated control animals (Fig. 4 K-O, Fig. 5 K-O). Brightfield imaging confirmed these findings as S129+ neurons were observed throughout the enteric tissue (Sup. Fig. 3). Again, as was seen in A53T treated animals, following PFF injections a significant amount of S129+ α -syn was found in a punctate (i.e. aggregate) pattern and no S129 immunoreactivity was seen in saline controls. We also observed diffuse S129 α -syn immunoreactivity throughout aggregate containing cells (e.g. Fig. 4 B-D, Fig. 5 B-D). Immunofluorescent staining with a pan- α -syn antibody confirmed what was observed using the S129 antibody; Both PFF and monomer treatment was associated with both diffuse and punctate staining patterns throughout the neurons (Sup. Fig. 4,5). This was also confirmed using brightfield immunohistochemistry as PFF and monomer treatment (Sup. Fig. 3 G,H, K, L) was associated with a significant degree of both punctate and diffuse immunoreactivity. In contrast, endogenous α -syn expression was much less pronounced in saline treated animals, and no

aggregated α -syn was seen in these animals using DAB immunohistochemistry (Sup. Fig. 3 M-P).

Comparison between treatments

A qualitative histological analysis between the various treatment groups revealed a pattern of α -syn immunoreactivity that was present regardless of histological methodology. The extent of aggregation suggested a pattern of A53T>PFF>monomer with regards to the degree of aggregated α -syn present (e.g. Fig. 4). In addition, a qualitative analysis suggests that the presence of aggregates within a neuron corresponded to a concomitant increase of nonaggregated α -syn within that same cell (e.g. Fig. 5). With either treatment there was no overt loss of HuC+ neurons within the ganglia surveyed.

CNS histology-NHP

As for the rat 12-month time-point, no S129 α -syn immunoreactivity was seen in any nucleus across the neuraxis in the NHP, including the DMN (Fig. 6). In contrast, robust staining was seen in positive control tissues from other studies in which rAAV- α -syn or PFF's were injected into the monkey CNS (Sup. Fig. 7).

Discussion

Here we demonstrate that the inoculation of pathological α -syn into the enteric nervous system of the rat results in a transient GI phenotype, with persistence of α -syn pathology in enteric neurons for as long as one year following the treatment in both rats and NHPs. Moreover, we observed some α -syn pathology in the brainstem of PFF treated rats one month following the

injection. However, this pathology was not observed at later time points in either species, suggesting clearance of pathological α -syn and a lack of any significant spread.

Enteric α -syn Pathology Causes GI Dysfunction

Numerous reports have described the presence of (pathological) α -syn in the ENS of Parkinson's disease patients (Wakabayashi *et al.*, 1988; Wakabayashi *et al.*, 1990; Braak *et al.*, 2006; Beach *et al.*, 2010). However, it is yet not clear whether this enteric α -syn is directly responsible for the GI dysfunction seen in Parkinson's disease patients, or whether it is an unrelated epiphenomenon. α -syn transgenic mice exhibit a GI phenotype (Kuo *et al.*, 2010; Hallett *et al.*, 2012; Wang *et al.*, 2012; Farrell *et al.*, 2014). However, because of the widespread α -syn brain and spinal cord pathology it is not possible to draw the conclusion that enteric α -syn pathology is the root cause of GI dysfunction in these mice. Herein, we describe, for the first time, that pathological forms of α -syn delivered directly to the ENS cause a GI phenotype in the form of reduced colonic motility. Although the DMN certainly is a crucial component of normal GI function (Pagani *et al.*, 1985; Travagli and Anselmi, 2016) the sporadic α -syn pathology observed, and the lack of neuronal loss, argues against a CNS-mediated component to the GI phenotype that was observed in this study.

Data collected from Parkinson's disease patients suggest that enteric α -syn pathology does not correlate with neuronal loss in the ENS (Annerino *et al.*, 2012); accordingly, it is unlikely that neuronal loss is the root cause of GI dysfunction. Our long-term data collected from NHPs are in agreement with this observation as we did observe profound α -syn pathology (i.e. "Lewy-like" pathology in surviving neurons) without any overt enteric neuron loss. This suggests that the aggregates themselves ultimately serve to induce functional changes in enteric

neurons. We have previously postulated that α -syn aggregation may act to sequester soluble (i.e. functional forms) of α -syn, depleting the cell of this protein (Benskey *et al.*, 2016a). This phenomenon has been observed in a number of *in vitro* and *in vivo* models (Cali *et al.*, 2012; Osterberg *et al.*, 2015). Interestingly, our data suggest that aggregation itself does not correlate well with a loss of soluble α -syn. Rather, it appeared as though α -syn aggregation was correlated with increased levels of diffusely disbursed α -syn within the soma. Moreover, S129+ synuclein was similarly observed in a diffuse pattern in enteric neurons both with immunofluorescent and immunohistochemical imaging. It is possible that the pronounced aggregation of α -syn resulted in a compensatory increase in expression of endogenous α -syn, ultimately resulting in a functional phenotype.

Persistent α -syn Pathology of the ENS does not Result in Sustained Pathology of the CNS

A central tenet of the prion hypothesis of α -syn suggests that pathological forms of α -syn exists in a perpetual cycle of 1) neuronal uptake, 2) retrograde transport within the neuron, 3) seeding of endogenous α -syn to pathological forms within the recipient cell, and 4) subsequent propagation of the pathology to recipient cells (Luna and Luk, 2015). This theory is very powerful as it holds great explanatory power in regards to the temporal presentation of α -syn pathology and symptoms in Parkinson's disease patients (Braak *et al.*, 2003a). In our approach we observed uptake of recombinant α -syn protein into enteric neurons (rat and NHP), aggregate formation in enteric neurons (rat and NHP), and shortly following the injection we observed pathological α -syn in the rodent brainstem, suggestive of uptake and transport of the protein via the vagal nerve. This finding is in agreement with prior findings which have described transport of α -syn via the vagus nerve from its injection site in the stomach wall to the DMN (Holmqvist *et al.*, 2014). These studies however, did not look at the later time points evaluated herein (6 days

was the longest time point evaluated in the prior work). The difference in pathology seen between these 2 studies is thus likely the result of a temporal pattern of clearance of the retrogradely transported α -syn which originated from the single injection bolus and was taken up directly by vagal afferents within the colon. It is unlikely that the pathological α -syn observed in brainstem nuclei originated from enteric neurons and was subsequently propagated to CNS neurons (i.e. it is unlikely that α -syn was first endocytosed by enteric neurons and that pathological α -syn was thereafter transmitted to the vagus nerve from these neurons) since we observed no sustained brainstem pathology 12 months after the injections, despite the persistence of robust enteric α -syn pathology over time. Along those lines, because we observed no pathology in Parkinson's disease -relevant structures, and because the limited α -syn pathology that was observed in the CNS diminished over time, our findings argue that the transported pathology is not sufficient to induce, nor sustain, a Parkinson's disease -like pathology in the brain (e.g. induce propagation to the substantia nigra). Taken together, our findings thus suggest that α -syn pathology in enteric neurons is not the source of CNS pathology in disease.

Our findings pose several alternate possibilities to the ENS-CNS prion hypothesis of Parkinson's disease. 1) A single bolus of pathological α -syn in the ENS is not sufficient to facilitate propagation. This is an unlikely scenario as overexpression (i.e. continuous production of pathological α -syn) also failed to elicit CNS pathology. Moreover, the concentration of PFFs injected in this study mirrored that in other studies performed in the PNS (Holmqvist *et al.*, 2014) or the CNS (Luk *et al.*, 2012a; Paumier *et al.*, 2015). 2) Additional factors are required for the propagation of Parkinson's disease -like α -syn pathology. Our studies outlined herein did not incorporate crucial factors such as aging (main risk factor for Parkinson's disease (Collier *et al.*, 2011)), a concurrent disease state, or systemic issues such as inflammation (Lema Tome *et al.*,

2013; Kelly *et al.*, 2014). It is possible that future studies incorporating additional factors such as these will be able to elicit propagation of α -syn from the ENS to the CNS. 3) ENS-CNS spread takes place, but over a much longer time span. It is thus important to note that it may be difficult to faithfully model the natural progression of human synucleinopathies in animal models. Although we utilized a long-time survival in this study, one year may not be representative of the time span required for α -syn to spread in Parkinson's disease. Indeed, enteric dysfunction can be observed many years prior to CNS pathology (Ross *et al.*, 2012), and it is possible that the spread and progression of pathogenic α -syn requires decades as opposed to years. One study found that a full truncal vagotomy is associated with a lower risk of developing Parkinson's disease (Svensson *et al.*, 2015), although subsequent analyses of the same data set, as well as other studies failed to replicate this finding (Tysnes *et al.*, 2015; Liu *et al.*, 2017). Nevertheless, the finding that the brainstem pathology that we observed early following the injections disappeared without spreading speaks against this point. 4) Propagation from the peripheral nervous system to the CNS does not take place; instead enteric and central α -syn pathology are two distinct phenomena which occur in Parkinson's disease patients on different time lines. Perhaps they are related to a common intrinsic state of susceptible neurons in Parkinson's disease but they cannot be directly connected. From this point of view, it is likely that Parkinson's disease, and the synucleinopathy observed in Parkinson's disease, represent a systemic disease state affecting many tissues throughout the body, and that any differential susceptibility of separate groups of neurons to α -syn pathology may represent differential functional reserves between neurons/circuits affected in Parkinson's disease (Engelender and Isacson, 2017). This is supported by pathological reports showing that there is rarely a perfect progression of α -syn pathology from ENS to brain (or within the brain, pathology does not always progress in an

ascending pattern) (Beach *et al.*, 2009; Beach *et al.*, 2010). Rather, more often than not, α -syn pathology is observed in many places concomitantly 5) Propagation of α -syn pathology is a receptor-mediated event, and enteric neurons do not express these receptors. Recent advances in the field has identified transmembrane proteins which are responsible for the endocytosis of PFF; lymphocyte-activation gene 3 (LAG3) and transmembrane protein 9 superfamily member 2 (TM9SF2) (Mao *et al.*, 2016; Wadman, 2016). Little is known about TM9SF2, and although LAG3 is expressed in CNS neurons, it is unclear whether this protein is also expressed in the ENS or on distal vagal nerve endings. Indeed, recent findings from the Di Monte group indicated that α -syn pathology (via AAV- α -syn overexpression) can spread from midbrain nuclei to the DMN throughout the vagal innervation of the ENS, but no pathology was observed in enteric neurons despite high levels of ectopic α -syn in the vagus nerve (Ulusoy *et al.*, 2016).

Alternatively, it is possible that enteric neurons in the colon do not express high enough levels of endogenous α -syn to effectively seed aggregates. It is clear from studies in the CNS that PFF-mediated pathology is dependent on endogenous expression of α -syn, where PFF-induced pathology is not observed in neurons of α -syn knockout mice (Luk *et al.*, 2012b; Volpicelli-Daley *et al.*, 2014). However, α -syn expression is present throughout the gastrointestinal tract in both humans and rodents, including the colon (Phillips *et al.*, 2013; Barrenschee *et al.*, 2017), thus a lack of endogenous α -syn is likely not the cause of our findings.

What is the role of enteric α -syn pathology in Parkinson's disease?

One important outcome of this study is the finding that pathological α -syn, almost exclusively localized within enteric neurons, induced a GI phenotype in the absence of overt neurodegeneration. Although the reduction in colonic motility was transient in nature, this phenotype was likely an early response to the protein injections, and the recovery in colonic

motility may be the result of the dwindling pathology that we observed. Indeed, in parallel studies in rodents we have found that continuous α -syn overexpression (via AAV) in enteric neurons results in long-term dysmotility (Benskey *et al.*, 2015a). Our finding is not entirely surprising as there is no loss of enteric neurons observed in Parkinson's disease (Annerino *et al.*, 2012). Importantly, the induction of α -syn pathology in non-dopaminergic neurons in the CNS can have profound effects on neuronal function and neurotransmission, without neurodegeneration (Caudal *et al.*, 2015; Alvarsson *et al.*, 2016). The precise role of (pathological) α -syn in GI function remains unknown and should be a focus of future research efforts.

Equally important to address is the genesis of α -syn pathology in the ENS in Parkinson's disease. As mentioned above, the aging neurons of Parkinson's disease patients may exhibit properties making them prone to α -syn aggregation regardless of anatomic location; but again, these events are not necessarily connected. For instance, increased levels of expression, or a shift in the subcellular localization of α -syn to the soma, will enhance cellular crowding and promote aggregation (Uversky *et al.*, 2002). In addition, a significant focus has been placed on the microbiota and other extrinsic factors affecting the state of α -syn in enteric neurons. For instance, recent findings drew the correlation between specific GI microbiota and CNS disease progression in an α -syn transgenic mouse (Sampson *et al.*, 2016). Although the underlying cause of this phenomenon is yet to be elucidated and likely multifactorial; the authors demonstrated that the increased production of short-chain fatty acids mediated, at least in part, the CNS pathological process in these mice (Sampson *et al.*, 2016). Importantly, short-chain fatty acids can cross the blood brain barrier. Thus, although there may not be a direct *physical* connection

between ENS and CNS α -syn pathology in Parkinson's disease, these pathologies may be linked via "endocrine-like" mechanisms.

Conclusion

In conclusion, our observations suggest that spread of α -syn pathology from enteric neurons to the brain is extremely limited in its scope, and not sufficient to sustain pathological spread of α -syn throughout the CNS on its own. Our findings give pause to the idea that the ENS is the original source of CNS pathology in Parkinson's disease, or indicate that factors in addition to exposure to pathological α -syn may be required for efficient transmittal to the CNS. Nevertheless, our findings do not diminish the importance of the ENS in the Parkinson's disease disease progress. Rather, we observed that enteric α -syn pathology is sufficient to elicit colonic dysmotility without the influence of central input. Given that GI symptoms are so prevalent in Parkinson's disease, our findings thus emphasizes the importance of focusing on enteric neuron function as treatments for these devastating comorbidities are being therapeutically investigated.

Author contributions

FPM and **JHK** conceived, and oversaw the execution, of these experiments. **KCL** provided recombinant protein and PFFs. **JG** performed rat injections and colonic motility assays. **MJB** performed the CNS histology of rats. **AG** and **AOM** performed enteric histology of rats. **IMS** assisted with vector preparation and *in situ* vector analysis. **JRP** performed the EM. **NCK** and **RY** performed all enteric analyses of NHP tissue.

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Financial Disclosure/Conflict of Interest

None

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Figure Legends

Fig. 1 Colonic inoculations of α -syn results in a transient gastrointestinal phenotype but persistent gastrointestinal pathology Gastrointestinal motility was evaluated monthly in rats following treatment by measuring fecal water content (**A**) and total fecal output (**B**). **A**) Both PFF (black bars, n = 15) and monomer (grey bars; n = 15) treated animals exhibited a significant decrease in fecal water content one month after the treatment as compared to saline treated controls (red bars; n = 14), and reduced fecal output was seen in PFF treated animals three months after the inoculation (**B**). However, GI dysfunction normalized between groups over time (**A,B**). Endogenous α -syn immunoreactivity following PFF (**C-E**), monomer (**F-H**), or saline treatment (**I**) reveal a persistent punctate staining pattern that persists over 6 months in PFF treated animals, and with a decline between 6 (**D**) and 12 (**E**) months. **F-H**. The same pattern of immunoreactivity was observed to a lesser degree in monomer treated animals. **I**. No significant α -syn immunoreactivity was seen in saline treated controls. Black markings in upper right corner

indicate the tattoo that was placed proximally to the injection zone. Error bars in **A** and **B** represents the mean + SD. * $p=0.012$ versus saline, # $p<0.05$ versus saline, ** $p<0.05$ versus all groups. Scale bar in **H** = 100 μm and applies to **C-I**. Scale bar in inset **H** = 20 μm and applies to insets **C-H**. MON=monomer. SAL= saline.

Fig. 2. A single inoculation of α -syn fibrils and monomers in the rat ENS results in the persistence of insoluble, Proteinase-K resistant, α -syn aggregates. Rats received injections of either α -syn PFFs (**A-C**), α -syn monomers (**D-F**), or saline (**G**) to the descending colon. In order to determine whether the α -syn immunoreactivity shown in Figure 1 represents “lewy-like” insoluble aggregates, enteric tissue was also analyzed for Proteinase-K resistant α -syn immunoreactivity. Proteinase-K resistant α -syn aggregates persisted within the ENS for up to one year following PFF (**A-C**) or α -syn monomer (**D-F**) delivery. No proteinase-K resistant α -syn was seen in saline treated controls (**G**). Scale bar in **A**=100 μm and applies to **A-G**. Scale bar in inset **A**=10 μm and applies to insets **A-G**.

Fig. 3 Colonic Injections of α -syn PFFs results in transient and minor α -syn accumulation in the brainstem Panels **A-I** show representative photomicrographs of serine 129 phosphorylated α -syn staining at the level of the DMN in 1, 6, and 12 month animals treated with saline (**A,D,G**), α -syn monomer (**B, E, H**) or α -syn PFF (**C,F, I**), respectively. Arrowheads in panel **C** indicates aggregated α -syn (S129+) within the DMN at one month following colonic injections of α -syn PFFs. Arrowheads in Panel **J** indicate positive detection of S129 phosphorylated α -syn within the locus coeruleus one month following injection of α -syn PFF. **Panel K** shows tissue used as a positive control for serine 129 phosphorylated α -syn immunoreactivity. Representative photomicrographs show S129+ aggregated α -syn within

neurons of the SNc, one month following striatal injections of α -syn PFFs. Panel **L** shows quantification of neurons in the DMN using the pan-neuronal marker HUC. Columns in Panel **L** represent the mean number of HUC+ neurons (normalized per sections counted) + 1 SEM (n=3-4/group). Panels **M&N** show representative HUC staining at the level of the DMN in saline and PFF treated animals, 12 months post-injection (representing the time point at which we would expect to see the greatest degree of toxicity). Abbreviations: dorsal motor nucleus of the vagus (DMN), Area postrema (AP), central canal (CC), locus coeruleus (LC). Scale bar in the low and high magnification images of Panel **I** represent 250 μ m and 50 μ m respectively, and apply to panels **A-H** and **L & M**. Scale bars in low and high magnification images in Panel **J** represent 500 μ m and 25 μ m respectively. Scale bars in low and high magnification images in Panel **K** represent 250 μ m and 25 μ m, respectively.

Fig. 4 Alpha-synuclein pathology persists in the non-human primate colon one year

following the inoculation Sections from the colon of NHPs treated with PFF (**A-E**), α -syn monomer (**F-J**), Saline (**K-O**), rAAV-A53T (**P-T**), and rAAV-GFP (**U-Z**) where double-stained with the pan-neuronal marker HuC/D (red) and serine 129 phosphorylated α -syn (green). **A-E**. PFF treatment was associated with the presence of intraneuronal S129+ α -syn inclusions, which was also observed in monomer treated subjects (**F-J**). No S-129+ α -syn was observed in enteric neurons in saline treated animals (**K-O**). **P-T**. rAAV-mediated overexpression of human α -syn A53T gave rise to a high degree of α -syn aggregates in enteric neurons; no S129 α -syn was seen in the AAV-GFP treated controls (**U-Z**). White arrows (**D, I, S**) indicate examples of S129+ neurons. Box in **A, F, K, P, U** outlines the area of magnification in **B-D, G-I, L-N, Q-S, V-Y**, respectively. Box in **D, I, N, S, Y** outlines the cell analyzed using confocal imaging in **E, J, O, T**, and **Z**, respectively. Scale bar in **U** = 100 μ m and also applies to **A, F, K**, and **P**. Scale bar in **Y** =

20 μm and also applies to B-D, G-I, L-N, Q-S, V-Y. Scale bar in Z = 10 μm and also applies to E, J, O, T.

Fig. 5 Alpha-synuclein pathology persists in the non-human primate stomach one year

following the inoculation Representative photomicrographs of sections from the stomach from NHPs treated with PFF (**A-E**), α -syn monomer (**F-J**), Saline (**K-O**), rAAV-A53T (**P-T**), and rAAV-GFP (**U-Z**). Dual label fluorescence of the pan-neuronal marker HuC/D (red) and serine 129 phosphorylated α -syn (green) reveal a significant degree of intraneuronal S129+ α -syn aggregates with PFF treatment (**A-E**) and α -syn A53T overexpression (**P-T**), and to a lesser degree in subjects injected with α -syn monomeric protein (**F-J**). No S129+ synuclein was observed in either saline (**K-O**) or rAAV-GFP (**U-Z**) treated controls. Box in A, F, K, P, U outlines the area of magnification in B-D, G-I, L-N, Q-S, V-Y, respectively. Box in D, I, N, S, Y outlines the cell analyzed using confocal imaging in E, J, O, T, and Z, respectively. Scale bar in U = 100 μm and also applies to A, F, K, and P. Scale bar in Y = 20 μm and also applies to B-D, G-I, L-N, Q-S, V-Y. Scale bar in Z = 10 μm and also applies to E, J, O, T.

Fig. 6 Lack of brainstem alpha-synuclein pathology in the non-human primate brain 12

months after the enteric inoculations. Low (**A**) and medium (**B**) power photomicrographs demonstrating the lack of serine 129 phospho- α -syn staining in a subject that received PFF injections in to the ENS of the stomach and colon 12 months prior. IV=fourth ventricle; XII=hypoglossal nucleus; DMN X= dorsal motor nucleus of the vagus nerve. Scale bar in A = 400 μm . Scale bar in B = 100 μm .

Supplementary Figure 1. EM characterization of PFFs. Electronmicrographs of mouse (A) and human (B) fibrils after sonication. Distribution of fibril length of mouse (C) and human (D) samples after sonication. Scale bar in B = 200 nm and also applies to A.

Supplementary Figure 2. Colonic injections of α -syn PFFs does not produce detectable α -syn pathology within the spinal cord at one month post-injection. Panels A-F show representative photomicrographs of serine 129 phosphorylated α -syn staining within the lumbar spinal cord of animals treated with saline (A-C) or α -syn PFF (D-F), one-month post-surgery. Panels (B-C) and (E-F) show high magnification images of the intermediolateral nucleus of the lumbar spinal cord, corresponding to the areas within the boxes in panels (A) and (D), respectively. Scale bar in panel F = 50 μ m and also applies to B, C, and E.

Supplementary Figure 3. Brightfield imaging of NHP enteric pathology. Because of the significant background seen with immunofluorescence, separate sections of monkey colon tissue was processed for immunohistochemistry in order to confirm the presence of aggregated α -syn. A. A53T treatment correlated with both dense and diffuse S129+ immunoreactivity. B. Increased magnification of area outline in A. C. Similarly, immunohistochemistry using an antibody against endogenous α -syn showed both an aggregated and diffuse staining pattern of α -syn. D. Increased magnification of area outline in C. Likewise, PFF (E) and monomer (I) treatment also resulted in the appearance of both aggregated and diffuse immunoreactivity of S129 phosphorylated α -syn (F and J. Increased magnification of area outline in E and I respectively) which was also reflected in the endogenous α -syn staining pattern (G, K). H, L. Increased magnification of area outlined in G, K respectively. M. No S129+ α -syn was seen in saline treated animals. N. Increased magnification of area outline in M. O-P. Saline treatment was associated with the presence of a modest amount of diffuse endogenous α -syn. P. Increased

magnification of area outline in **O**. Scale bar in **O** = 100 μm and also applies to **A**, **C**, **E**, **G**, **I**, **K**, **M**, **O**. Scale bar in **L** = 10 μm and also applies to **B**, **D**, **F**, **H**, **J**, **L**, **N**, **P**.

Supplementary Figure 4. Pan α -syn immunoreactivity of the NHP colon. Sections from the colon of NHPs treated with PFF (**A-D**), α -syn monomer (**E-H**), Saline (**I-L**), rAAV-A53T (**M-P**), and rAAV-GFP (**Q-T**) were double-stained with a pan-neuronal marker (HuC/D; red) and an antibody recognizing all species isoforms of α -syn (pan α -syn; green). As was seen with the S129 antibody, PFF treatment (**A-D**), α -syn monomer treatment (**E-H**), and rAAV-A53T delivery (**M-P**) all produced a staining pattern of both diffuse and aggregated α -syn. In contrast, only diffuse α -syn was present, albeit to a lesser degree than in α -syn treated subjects, in enteric neurons of animals treated with saline (**I-L**) or rAAV-GFP (**Q-T**). White arrows (**D**, **H**, **P**) indicate examples of neurons containing aggregated α -syn. Box in **A**, **E**, **I**, **M**, **Q** outlines the area of magnification in **B-D**, **F-H**, **J-L**, **N-P**, **R-T**, respectively. Scale bar in **Q** = 100 μm and also applies to **A**, **E**, **I**, **M**. Scale bar in **Y** = 20 μm and also applies to **B-D**, **F-H**, **J-L**, **N-P**, **R-T**.

Supplementary Figure 5. Pan α -syn immunoreactivity of the NHP stomach. Representative photomicrographs of sections from the stomach from NHPs treated with PFF (**A-D**), α -syn monomer (**E-H**), Saline (**I-L**), rAAV-A53T (**M-P**), and rAAV-GFP (**Q-T**). Dual label fluorescence of the neuronal marker HuC/D (red) and pan α -syn (green) reveal a significant degree of aggregated α -syn with PFF treatment (**A-D**) and α -syn A53T overexpression (**M-P**), and to a lesser degree in subjects injected with α -syn monomeric protein (**E-H**). Only a diffuse staining pattern of α -syn can be observed in saline (**I-L**) and rAAV-GFP (**Q-T**) treated controls. White arrows (**D**, **H**, **P**) indicate examples of neurons containing aggregated α -syn. Box in **A**, **E**, **I**, **M**, **Q** outlines the area of magnification in **B-D**, **F-H**, **J-L**, **N-P**, **R-T**, respectively. Scale bar in

Q = 100 μ m and also applies to A,E,I,M. Scale bar in Y = 20 μ m and also applies to B-D, F-H, J-L, N-P, R-T.

Supplementary Figure 6. Verification of GFP transduction. In order to confirm successful and persistent ENS neuronal transduction of rAAV, colon sections were stained for the GFP transgene. **A.** Low magnification image with the presence of a ganglion in the middle of the figure. **B.** Higher magnification of area outlined in **A**, showing GFP immunoreactivity in numerous cells throughout the ganglion. Scale bar in A = 100 μ m. Scale bar in B = 10 μ m.

Supplementary Figure 7. Positive control of NHP midbrain PFF pathology. Low (A) and medium (B) power photomicrographs demonstrating the serine 129 α -syn immunoreactivity in substantia nigra from a monkey that received human α -synuclein PFFs (the same as used in this study) in the striatum 12 months prior. Scale bar in A = 400 μ m. Scale bar in B = 100 μ m.

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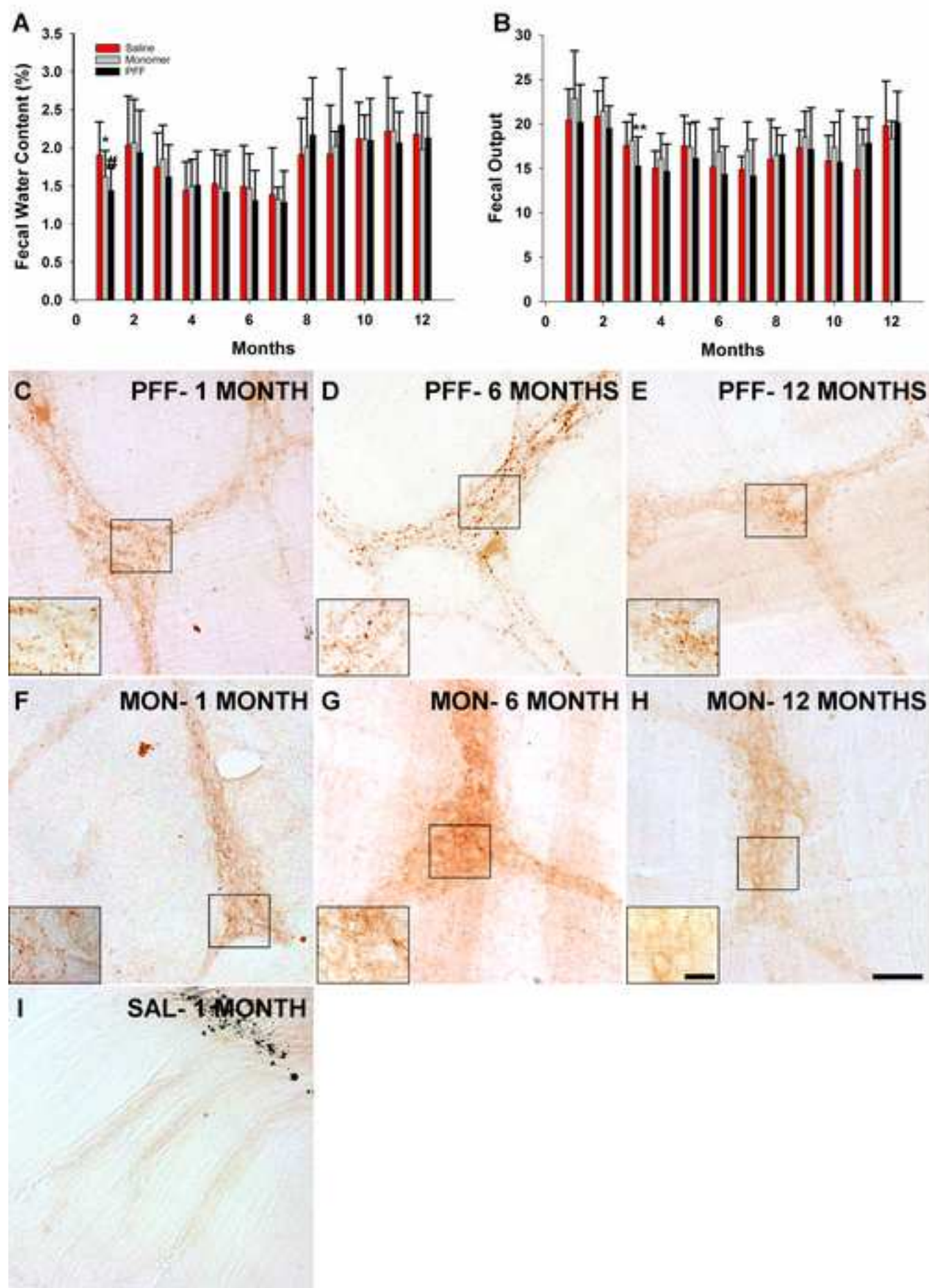


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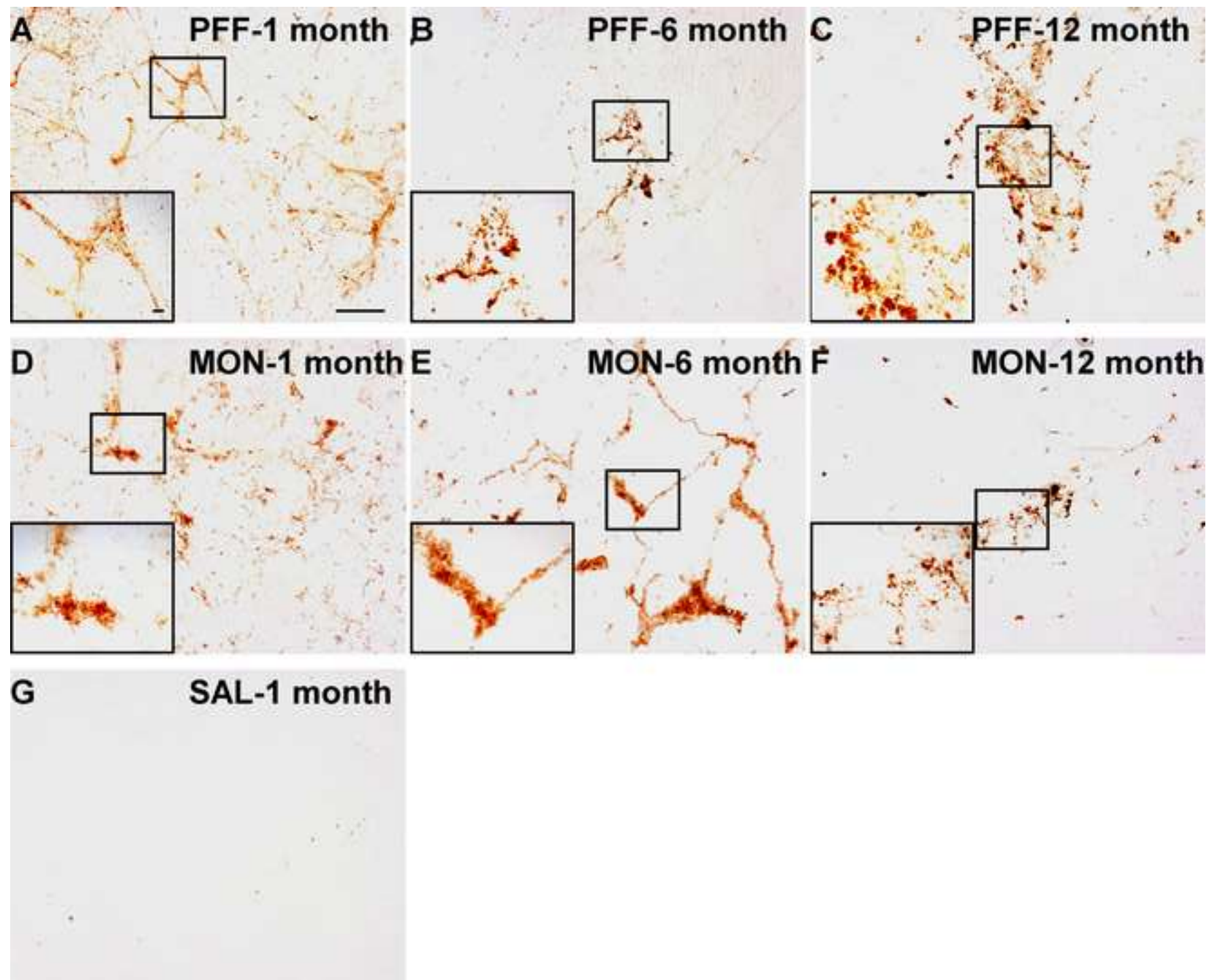


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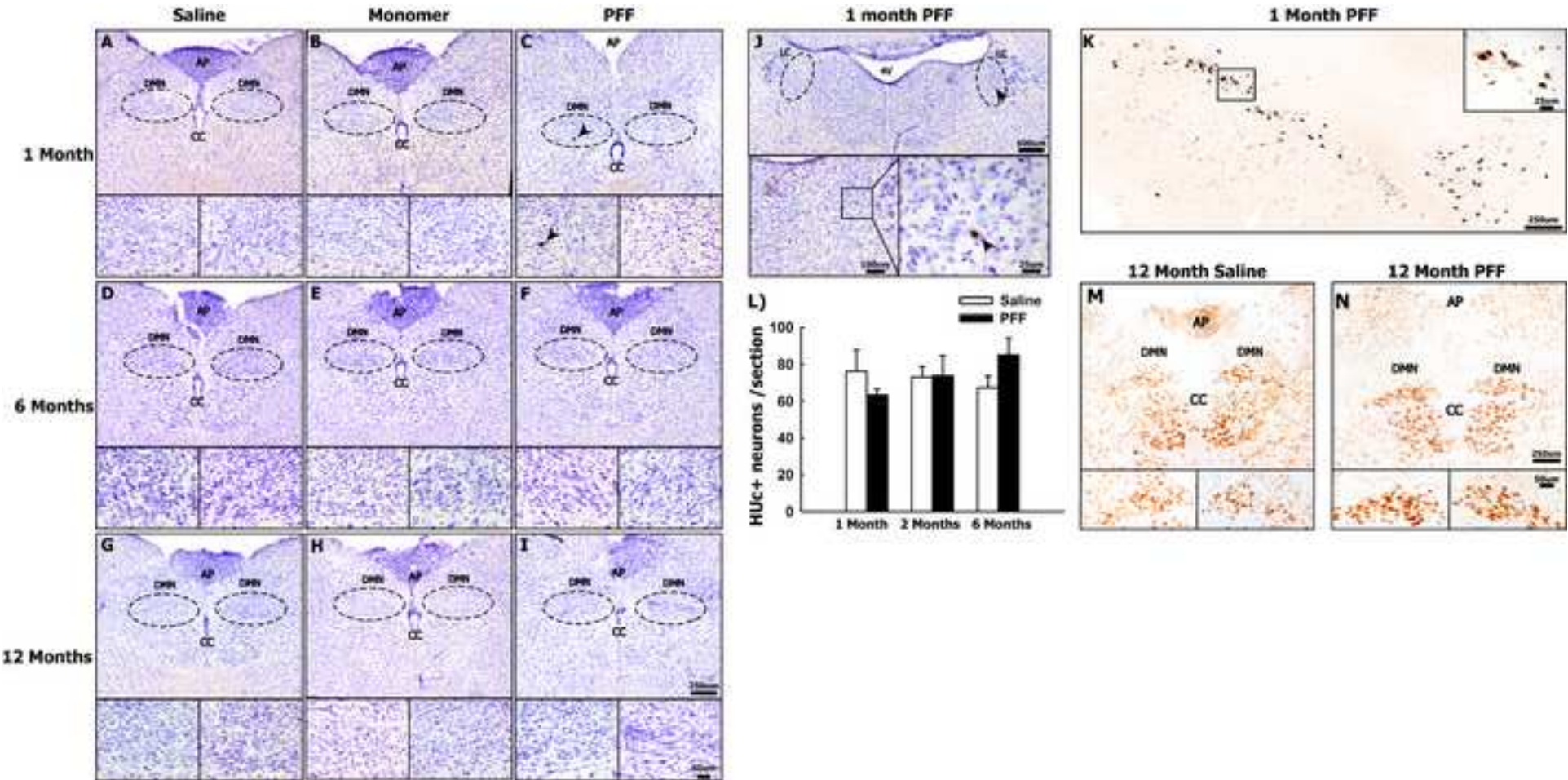


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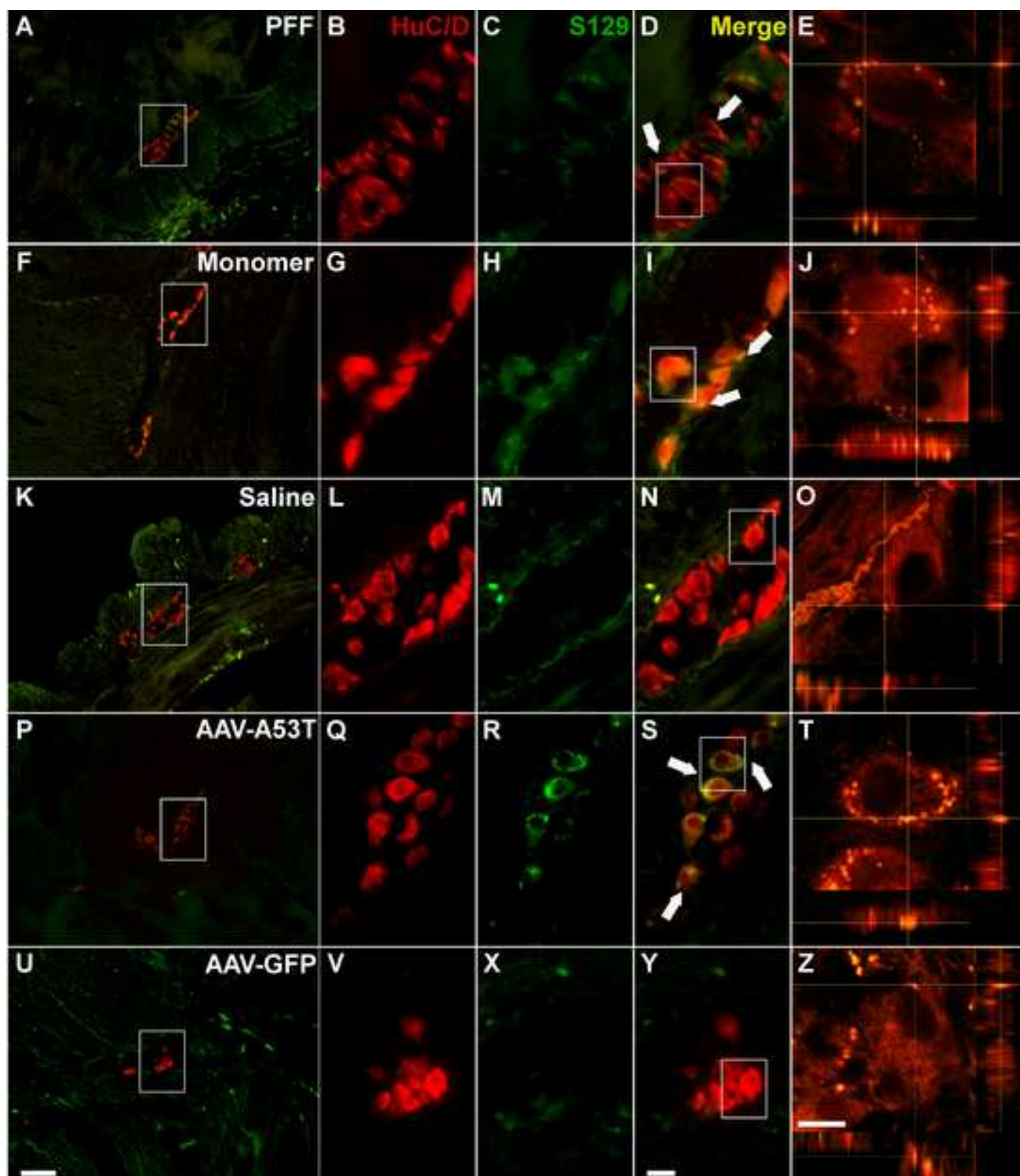


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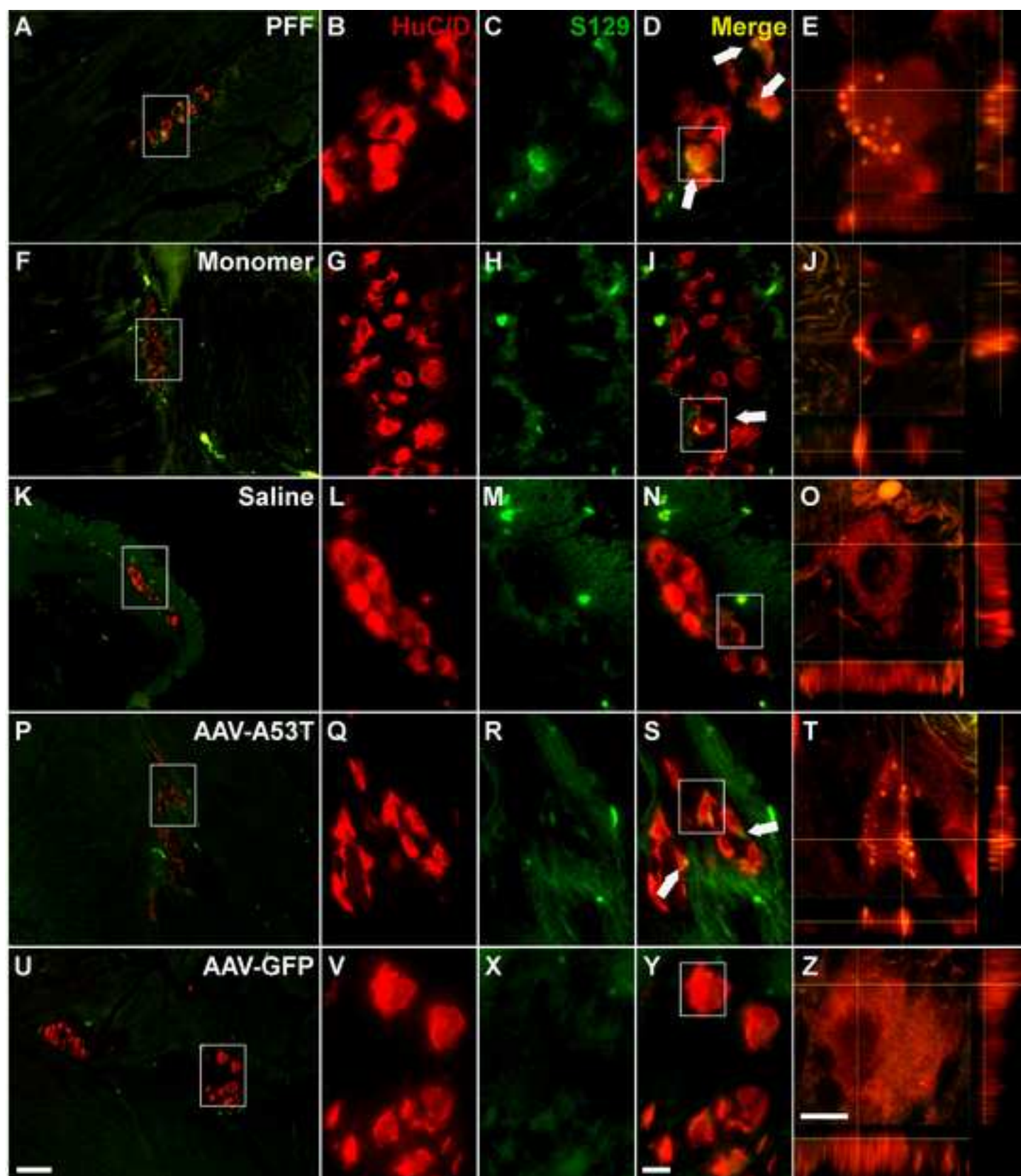
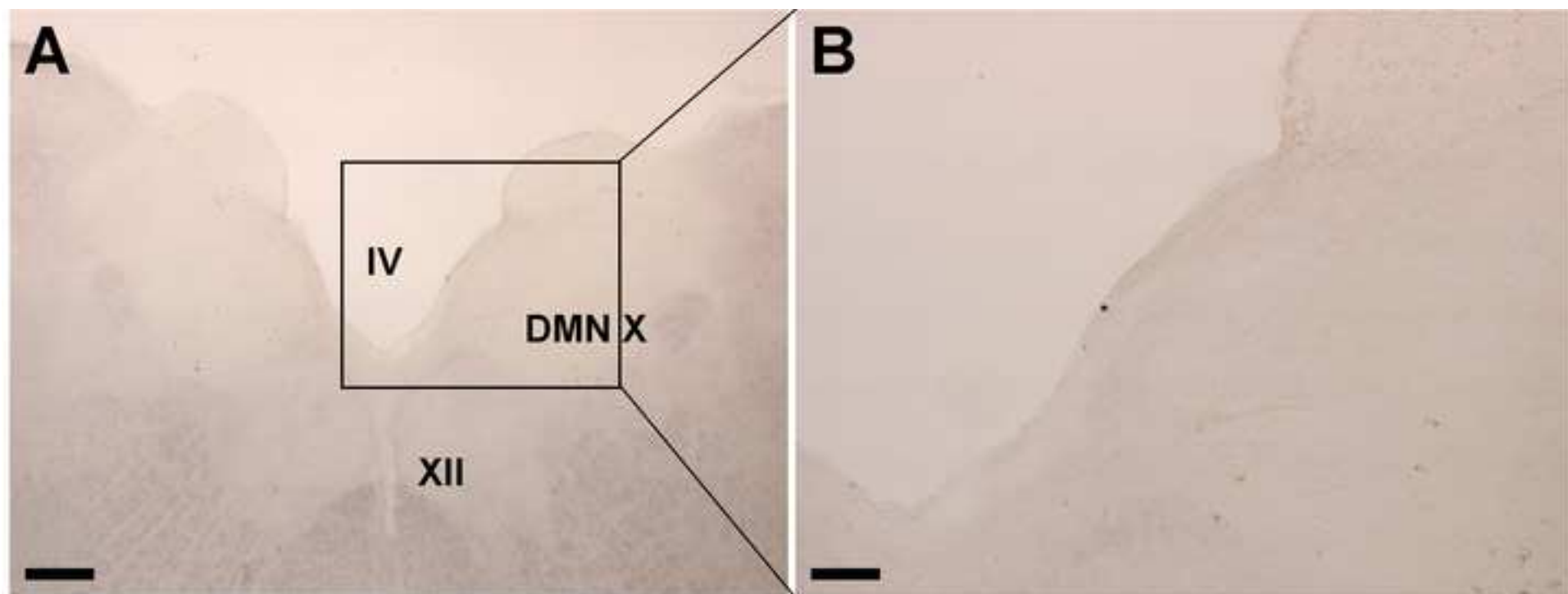


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