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TITLE: Functional Analysis of Nonmotor Symptoms from Regions Innervating the Locus Coeruleus in a Mouse Gut-Brain Alpha-Synuclein PFF Model

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14. ABSTRACT

The non-motor symptoms associated with Parkinson's disease have been largely overlooked, gaining only small recognition in the last decade. These NMS vary, encompassing both the psychiatric and the cognitive. The loss of noradrenergic neurons in the locus coeruleus precedes the loss of dopamine neurons in the SNc and to a greater extent. Like the SNc, the loss of LC neurons may result from the accumulation of alpha-synuclein, which is the main component in Lewy bodies and has been postulated to behave like a prion leading to the Braak's hypothesis. The Braak hypothesis has been expanded based on the concept that pathologic a-syn may initiate in the gastrointestinal tract and spread in a retrograde manner from the enteric nervous system (ENS) up the vagus to the dorsal motor nucleus of the vagus. Since there was no appropriate animal model of a-syn transmission from the ENS to the CNS, we developed a clinically relevant gut-brain a-syn preformed fibril (PFF) transmission mouse model that recapitulates the spread of a-syn according to Braak's model. Following our SoW, made the PFF and rabies virus during the first quarter and injected PFF into the mouse gut during the second. We were able to harvest some of the early injected mice before the university shutdown due to the COVID-19 pandemic. These mice showed phospho-syn staining the duodenum and pylorus of the mice but we saw little to no labeling in the brain, and thus we were unable to reproduce the transmission previously reported. Due to the shutdown, we were forced to euthanize the remaining gut-injected mice and keep only breeding pairs. After further investigation, we discovered that the reported dose of PFF had been incorrectly calculated resulting in only 20% of the reported PFF being injected. Secondly, the surgical anesthetic published (Isoflurane) was not the anesthetic used (Ketamine). Ketamine has been shown to have anti-inflammation properties, and it is believed that this reduction in inflammation and the 5-times the concertation of PFF that contributed to the previously reported results. This new information has been taken into account, and a new cohort of mice have been injected under these conditions and are expected to be harvested in mid-October. Once confirmed, additional cohorts will be injected for the rabies co-localization and *in vivo* calcium imaging studies.

15. SUBJECT TERMS
NONE LISTED

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

The non-motor symptoms (NMS) associated with Parkinson's disease have been largely overlooked, gaining only small recognition in the last decade. These NMS vary, encompassing both the psychiatric and the cognitive. The loss of noradrenergic neurons in the locus coeruleus (LC) precedes the loss of dopamine neurons in the SNc and to a greater extent. Like the SNc, the loss of LC neurons may result from the accumulation of alpha-synuclein (a-syn), which is the main component in Lewy bodies and has been postulated to behave like a prion leading to the Braak's hypothesis.

The Braak hypothesis has been expanded based on the concept that pathologic a-syn may initiate in the gastrointestinal tract and spread in a retrograde manner from the enteric nervous system (ENS) up the vagus to the dorsal motor nucleus of the vagus (DMV). Since there was no appropriate animal model of a-syn transmission from the ENS to the CNS, we developed a clinically relevant gut-brain a-syn preformed fibril (PFF) transmission mouse model that recapitulates the spread of a-syn according to Braak's model.

Our preliminary observations are that a-syn PFF injection into the duodenum and pylorus of wild-type mice leads to CNS synucleinopathy. Both Lewy bodies and Lewy neurites are found in the DMV followed by the LC in our gut-brain PFF model as is evident by posttranslational phosphorylation of a-syn at serine 129 (pSer129-a-syn). Furthermore, under normal physiological conditions, the locus coeruleus has been implicated in a number of behaviors including anxiety, depression, sleep disturbances, and memory formation. All of which are affected during Parkinson's disease. Our goal in to determine the LC behaviors effects of a-syn Gut-Brain transmission and identify other brain regions which may be involved.

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

Parkinson's disease, locus coeruleus, Non-motor symptoms, alpha-synuclein pre-formed fibril transmission, rabies circuit tracing, in vivo calcium imaging

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

What were the major goals of the project?

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

Major Task 1:

Subtask 1: Make alpha-synuclein PFF and Rabies-EGFPL10a. 1-3mo – 100% complete.
Subtask 2: Inject mice with PFF. 3-6mo – First cohort failed do to published PFF concentration error. Loss of mice do to COIVS-19 lab shutdown – 10% complete.
Subtask 3: Inject mice with AAV and rabies for co-localization with PFF. 6-9mo – this was step two using mice from Subtask 2. Mice show rabies labeling but because of the misreported PFF dose concentration, little to no phosphor-syn signal was detected – 0% complete.
Subtask 4: Perform histology, imaging, and analysis. 9-14mo – no useable mice/images. 0% complete.

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

We made the alpha-synuclein PFF and rabies virus during the first quarter and injected the PFF into the mouse gut during the second. We were able to harvest some of the early injected mice before the university shutdown due to the COVID-19 pandemic. These mice showed phospho-syn staining the duodenum and pylorus of the mice but we saw little to no labeling in the brain, and thus we were unable to reproduce the transmission previously reported. Due to the shutdown, we were forced to euthanize the remaining gut-injected mice and keep only breeding pairs. After further investigation, we discovered that the published concentration of PFF had been incorrectly calculated resulting in an injected dose of only 20% of that reported. Secondly, the surgical anesthetic published (Isoflurane) was not the anesthetic used (Ketamine). Ketamine has been shown to have anti-inflammation properties, and it is believed that this reduction in inflammation and the 5-times the concertation of PFF that contributed to the previously reported results. This new information has been taken into account, and a new cohort of mice have been injected under these conditions and are expected to be harvested in mid-October. Once confirmed, additional cohorts will be injected for the rabies co-localization and *in vivo* calcium imaging studies.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

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

The monthly seminars, which host top researchers preceded as expected until the COVID-19 pandemic forced the university to shut down. We have recently (Aug) started back the seminars virtually via Zoom. Attendance to the scheduled annual conference was also suspended due to COVID-19.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

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

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

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

Once we confirm alpha-synuclein gut-brain transmission using the correct PFF concentration and anesthetic in October, we will inject the appropriate cohorts of mice to complete year 1's tasks. Additionally, during the same period, we will inject additional cohorts for year 2's tasks since there is a 4-5 month aging period. During this aging period, we will process the brains from the first cohort. We also received new *in vivo* calcium imaging equipment that we will setup and calibrate.

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

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

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

Nothing to Report

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

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

Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

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

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

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

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

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

Nothing to Report

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

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

The change being made is the use of Ketamine + Xylazine in place of isoflurane for the anesthetic during the gut PFF injection.

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

The inaccurate reporting of the PFF concentration resulted in the mice from the first two cohorts being unusable. Provided our actions have corrected this issue, there is an estimated loss of time ~6mo. Due to the COVID-19 university shutdown and euthanasia of our research animals, we estimate an addition loss of 5mo. Provided the gut-brain transmission issue is resolved and the university remains open we estimate that we will need to request for a 6-8mo no-cost-extension

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

The inaccurate reporting of the PFF concentration resulted in the mice from the first two cohorts being unusable. This resulted in the expenditures of mice, animal housing, PFF, and surgical supplies. Due to the COVID-19 university shutdown and euthanasia of our research animals, we estimate an additional financial loss for mice and animal housing. However, due to COVID, the university has suspended all raises and promotions. As such, the PI's salary will be 20% less and the difference should cover the losses. However, at this time, the business office has not provided the end-of-year numbers.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

The only change is the use of Ketamine + Xylazine in place of Isoflurane as the anesthetic during the PFF injections.

Significant changes in use of biohazards and/or select agents

Nothing to Report

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

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

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

Nothing to Report

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

Nothing to Report

  Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as*

noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

Nothing to Report

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

Nothing to Report

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

Nothing to Report

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

Nothing to Report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

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

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: Adam A. Behensky
Project Role: PI
Researcher Identifier (e.g. ORCID ID): 0000-0003-0984-6013
Nearest person month worked: 12
Contribution to Project: Made viruses and PFF, performed surgeries and injections, analyzed data and wrote reports

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

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

Nothing to Report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

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

Provide the following information for each partnership:

Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

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

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

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

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

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