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TITLE: Novel Therapeutic Targeting of Neurotoxic Gut Microbiota-Derived Metabolites in Parkinson's Disease

PRINCIPAL INVESTIGATOR: Dr. Anumantha Kanthasamy, PhD

CONTRACTING ORGANIZATION: University of Georgia, GA 30602

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

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**4. TITLE AND SUBTITLE**

Novel Therapeutic Targeting of Neurotoxic Gut Microbiota-Derived Metabolites in Parkinson's Disease

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**5b. GRANT NUMBER**

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

Dr. Anumantha Kanthasamy, PhD

E-Mail:

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

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

University of Georgia
Athens, GA 30602

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

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## 14. ABSTRACT

Parkinson's disease (PD) is a heterogeneous neurodegenerative disorder affecting an estimated seven to 10 million people worldwide. While its exact etiology remains largely unknown, PD is likely to be multifactorial with contributions from both genetic and environmental factors. Recent evidence suggests that the gut-brain axis plays a large role in the development of PD, and gastrointestinal dysfunction and microbiome dysbiosis have been linked to the onset and progression of PD pathology. Using advanced microbial sequencing studies in the gut microbiomes of PD patients, we recently uncovered that the microbial pathways for synthesis of a neurotoxic chemical, trimethylamine (TMA) was highly elevated in PD patients. Microbiota-derived TMA is metabolized in the liver to trimethylamine N-oxide (TMAO), which enters systemic circulation and crosses the blood brain barrier and has been shown to accelerate the rate of α-synuclein (αSyn) fibril formation. Additionally, microbial TMA can also be converted into toxic metabolites formaldehyde (HCHO) and ammonia (NH3). Our preliminary studies also found significantly elevated plasma TMAO and HCHO production in PD patients. Our subsequent mechanistic studies further revealed that TMAO treatment promotes αSyn aggregation and stabilize its conformational changes and triggers inflammasome activation. Our proposed studies will test the following 3 objectives. In Objective 1, we will identify the TMA-generating bacterial species and strains that are elevated in human PD and establish the link between neurotoxic TMAO and HCHO metabolites, aggregated αSyn load and gut and peripheral inflammatory markers. In Objective 2, we will evaluate the therapeutic potential of pharmacological inhibitors of bacterial TMA production for disease modification in PD using well-established transgenic and humanized germ-free mouse models of PD. In Objective 3, we will evaluate the translational potential of the clinically tested, healthy probiotic strain *E. coli* Nissle in restoring gut microbial homeostasis and mitigating bacterial TMA production, αSyn aggregation and proinflammatory cascades in PD mouse models. Overall, we anticipate that our study will provide the basis for developing new treatment strategies targeting TMAO-mediated pathological processes for disease modification in PD.

| 15. SUBJECT TERMS | | | | | |
|---|---|---|---|---|---|
| Trimethylamine (TMA), trimethylamine-N-oxide (TMAO), Real-time quaking-induced conversion (RT-QuIC) assay, alpha(α)-Synucleinopathy, Parkinson's disease, biomarker, A53T transgenic animal model, E. coli strain Nissle 1917, L-DOPA, microbial dysbiosis, inflammatory bowel disease (IBD). | | | | | |
| **16. SECURITY CLASSIFICATION OF:** | | | **17. LIMITATION OF ABSTRACT** | **18. NUMBER OF PAGES** | **19a. NAME OF RESPONSIBLE PERSON** USAMRDC |
| **a. REPORT** | **b. ABSTRACT** | **c. THIS PAGE** | UU | **15** | **19b. TELEPHONE NUMBER** *(include area code)* |
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**Standard Form 298 (Rev. 8-98)**
**Prescribed by ANSI Std. Z39.18**

## TABLE OF CONTENTS

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

The research summarized in this annual report addresses the FY21 PRP Focus Area pertaining to the role of environmental exposures and gene-environment interactions in prodromal or clinically diagnosed Parkinson's disease (PD). PD is a complex progressive neurodegenerative disease characterized by cardinal motor impairment and α-synucleinopathy. Currently, more than one million Americans suffer from PD, and military personnel are at higher risk of developing PD than are civilians because they are often exposed to a wide range of toxins in the unique circumstances of their service and deployments. A wealth of emerging evidence suggests that disturbances in the gut-microbiome-brain axis including gastrointestinal (GI) dysfunction and microbiome dysbiosis play a critical role in the pathogenic mechanisms underlying PD. Consistent changes in gut microbiome and GI functions have been demonstrated in PD patients in multiple independent cohort studies. Intriguingly, recent studies from our laboratory and others have revealed that the microbiome of PD patients produces significantly higher levels of trimethylamine (TMA), the precursor of trimethylamine-N-oxide (TMAO) that is known to accelerate the rate of α-synuclein (αSyn) fibril formation. Supporting this, we also found significantly elevated plasma TMAO and formaldehyde production in PD patients compared to age-matched healthy controls. Our in vitro mechanistic studies further demonstrated that TMAO treatment stabilizes/promotes αSyn aggregation, triggers inflammasome activation and leads to GBA1 dysregulation. In light of the myriad military and civilian applications, whether microbiota-derived TMA acts as a potential trigger and facilitator of PD pathology along the gut-brain axis and whether targeting TMAO could be a viable therapeutic approach for PD have been of huge research interest. Thus, this project aims to elucidate the functional consequences of TMA and its downstream neurotoxic metabolites in both animal models of PD and human PD patients. Our novel hypothesis is that chronic gut microbial dysbiosis in PD generates the neurotoxic metabolic precursor TMA, which triggers and augments PD neuropathology through mechanisms involving α-synuclein aggregation, GBA1 dysregulation and NLRP3 inflammasome activation by its metabolites TMAO and FA. Thus, therapeutic inhibition of microbial TMA generation provides a radically new treatment approach by which to achieve disease modification in PD. We plan to identify the TMA-generating bacterial species and strains that are elevated in human PD and establish the link between neurotoxic TMAO and HCHO metabolites, aggregated αSyn load, and the gut and peripheral inflammatory markers. Afterward, we plan to evaluate the therapeutic potential of pharmacological inhibitors of bacterial TMA production for disease modification in PD using well-established transgenic and humanized gnotobiotic mouse models of PD. Finally, we plan to evaluate the translational potential of the clinically tested, healthy probiotic strain *E. coli* Nissle in restoring gut microbial homeostasis and mitigating bacterial TMA production, αSyn aggregation and proinflammatory cascades in PD mouse models. Successful completion of the proposed research will provide the basis for developing new treatment strategies aimed at reducing TMAO-mediated pathology in PD to slow or halt disease progression. Results from our studies will also strengthen the in vivo mechanistic links between elevated TMAO and key pathological processes involved in PD progression.

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

Trimethylamine (TMA), trimethylamine-N-oxide (TMAO), Real-time quaking-induced conversion (RT-QuIC) assay, alpha(α)-Synucleinopathy, Parkinson's disease, biomarker, A53T transgenic animal model, *E. coli* Nissle 1917, L-DOPA, microbial dysbiosis, inflammatory bowel disease (IBD).

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.*

**Statement of work for Site 1:** University of Georgia, GA

**Major Task 1:** Objective 1, 1-42 months.
Identify the TMA-generating bacterial species and strains that are elevated in human PD and establish the link between neurotoxic TMAO and HCHO metabolites, aggregated αSyn load and gut and peripheral inflammatory markers.

**Subtask 1:** 42-48 months.
Assessment of aggregated αSyn load in human PD and healthy control skin samples.

**Major Task 2:** Objective 2, 12-42 months.
Evaluate the therapeutic potential of pharmacological inhibitors of bacterial TMA production for disease modification in PD using a well-established transgenic and humanized gnotobiotic mouse model.
**Subtask 1:** 12-18 months
IACUC and ACURO approvals to be transferred and completed for all animal work.

*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.*

Below are the details of the major activities performed as well as the significant progress and key outcomes achieved during this year.

**Optimization of the purification protocol for recombinant human αSyn protein.**

As recombinant monomeric human α-synuclein protein is needed for determining whether TAMO induces αSyn seeding activity, we generated the recombinant protein. Our protein purification protocol was adopted from Groveman et al. PMCID, with minor modifications. Briefly, WT αSyn was expressed in BL21 *E. coli* transfected with a pT7-7 expression plasmid. The bacterial mini culture was grown at 37°C for 8 h and transferred to pre-warmed 1-L LB broth with antibiotics along with overnight autoinduction reagents. Once the $OD_{600}$ reached 1.2 – 1.3, cells were pelleted by centrifugation at 4°C. The supernatant was discarded. Each pellet was resuspended in an osmotic shock buffer and rocked gently at room temperature and centrifuged. The supernatant was discarded, and pellets were placed on ice. Each pellet was gently resuspended and centrifuged, and the supernatant underwent acid precipitation by reducing the pH to 3.5 with HCl (a white precipitate appears, turning the solution white and cloudy). The solution contents were centrifuged, the supernatant's pH was adjusted to 7.5, and was then collected in a 150-mL sterile glass beaker. The beaker was placed on a stirrer and the solution was gently stirred as the pH was increased to 7.5 using 1 M NaOH. The tubes were centrifuged and the supernatant was concentrated from 15-20 mL using 3-kDa centrifugal filters. The precipitate at the bottom of the filter was resuspended, collected and filtered using a 0.22-µm syringe filter and applied to the FPLC for size-exclusion and anion-exchange chromatography as described below.

*Size-Exclusion Chromatography (SEC)*

Next, the sample was manually loaded into the 10-mL sample loop of the Bio-Rad NGC FPLC chromatography system and systemically loaded into the column using SEC buffer. At a flow rate of 1 mL/min, 2 column volumes (CV) of fresh SEC buffer were used to elute the target protein in 3-mL fractions. The fractions with a high absorbance peak were visualized using SDS page and Coomassie staining. Fractions with strong putative αSyn expression (bands around 14 kDa) were pooled and carried into anion-exchange chromatography.

*Anion-Exchange Chromatography*

Following SEC, 10 mL of the pooled fractions from size-exclusion chromatography was manually loaded into the NGC chromatography system and subsequently loaded into the anion-exchange column (HiPrep Q FF 16/10) using 15 mL of low-salt buffer. At a flow rate of 1 mL/min (the isocratic segment), 2 CV of 5% high-salt buffer was pushed through the column without fraction collection. This was followed by a fraction collection elution stage using a flow rate of 1 mL/min with a 6-CV gradient from 20% to 55% of high salt buffer with the collection of fractions. Fractions below the absorbance peak were collected for SDS-PAGE analysis. The Coomassie-stained gel confirmed the expression of αSyn. Fractions with strong αSyn expression were pooled and carried into dialysis. Aliquots were made in 1.5-mL low-protein-binding tubes and stored at -80°C. Schematic 1 shows a detailed graphic of the full in-house αSyn purification method. This method has yielded very high levels of ultra-pure recombinant human αSyn monomeric protein as determined by SDS-PAGE and Western blot. The quality of purified monomeric αSyn protein was confirmed by Dynamic Light Scattering (DLS), MALDI-TOF Mass Spectrometry, TEM and CD spectra. Our αSyn protein purification method demonstrates excellent purity and stability with high yields.

**Optimization of the αSyn Seed Amplification Assay (αSyn SAA) of human and mouse skin samples.**

The purified αSyn monomeric protein was then subjected to αSyn SAA or the RT-QuIC assay to detect pathological aggregates of αSyn in autopsy and biopsy PD and health control skin tissues from humans and mice as described in our prior publications (PMCID: PMC7749035). Furthermore, we optimized this ultra-sensitive and specific assay to detect pathological αSyn present in human and mouse skin tissues. We further optimized the ultra-sensitive assay for ionic strength optimization to enhance the fidelity of fmax between positive and negative controls. The combination of αSyn purification and ionic strength optimization in αSyn SAA resulted in high intermediate precision and repeatability in αSyn SAA. These results collectively demonstrate successful optimization and validation of the αSyn SAA. Experiments performed with optimized purified human αSyn monomer and optimized ionic strength of the reaction mixture in αSyn SAA of autopsy and biopsy human and mouse skin tissues resulted in precision and robustness compared to published αSyn SAA protocols. We will be using these standardized protocols for detecting pathological αSyn in skin samples from human and mouse tissues.

**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.*

| Nothing to report |
|---|

**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.*

**Major Task 1, Subtask 1 (42-48 month):** UGA IRB approval to receive blinded de-identified skin samples from PD and HC subjects from Partnering PI.

We will be assessing aggregated αSyn load using αSyn SAA in de-identified skin samples from 70 PD and 70 HC subjects in the months remaining. In this regard, we have submitted an approval letter from IRB at UGA to HRPO. This letter gives us permission to obtain de-identified skin tissues from 70 PD and 70 HC subjects from Partnering PI Dr. Richard Gordon's lab at Queensland University of Technology, Australia. The Partnering PI award is pending award transfer to Queensland University of Technology. We are ready to receive the first subset of blinded de-identified skin samples and assess them for aggregated αSyn load.

**Major Task 2: Subtask 1 (12-18 months):** IACUC and ACURO approvals for animal studies to be performed in the A53T αSyn Tg mouse model scheduled for 24-42 months.

In this regard, we have the IACUC protocol approved for animal studies proposed at UGA. We have submitted the abbreviated ACURO version to the Animal Care and Use Review Office. Once approved, we will begin animal studies.

***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).*

In the first year of the proposal, we achieved significant results that will have an impact on the studies proposed in Years 2-3.
1) Since αSyn monomer substrate is a key determinant of the αSyn fibrillar assay (RT-QuIC), we optimized the recombinant αSyn expression and purification system by utilizing an efficient plasmid vector for constitutive high-level expression of human wild-type αSyn in *E. coli* and by adopting an improved purification protocol by FPLC.
2) We then optimized the αSyn seed amplification assay (αSyn SAA) with αSyn ultra-purified as described above in human skin samples. Human skin samples used are control samples from our previously published work.

**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:*

| |
|---|
| *Nothing to Report* |

**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.*

**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.*

| |
|---|
| Nothing to report |

*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.*

Nothing to report

**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**

Nothing to report

**Significant changes in use of biohazards and/or select agents**

Nothing to report

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

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

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

  *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:*** | ***Anumantha G Kanthasamy*** |
| *Project Role:* | *PI* |
| *Researcher Identifier (era commons):* | **akanthas** |
| *Nearest person month worked:* | *1.0* |
| *Contribution to Project:* | *He contributed to the overall leadership, coordination and supervision of the project.* |

| | |
|---|---|
| ***Name:*** | ***Arthi Kanthasamy*** |
| *Project Role:* | *Investigator* |
| *Researcher Identifier (ERA commons):* | ***arthik*** |
| *Nearest person month worked:* | *0.5* |

| | |
|---|---|
| *Contribution to Project:* | *She assisted in the interpretation of protein purification and αSyn RT-QuIC assays.* |
| ***Name:*** | ***Vellareddy Anantharam*** |
| *Project Role:* | *Investigator* |
| *Researcher Identifier (ERA commons):* | **vellareddypi** |
| *Nearest person month worked:* | *0.77* |
| *Contribution to Project:* | *He was responsible for supervising the training of laboratory personnel and serves as liaison in assuring our lab's compliance with EHS, IACUC, IBC and IRB. He also assisted the PI in preparing the annual report.* |
| ***Name:*** | ***Huajun Jin*** |
| *Project Role:* | *Associate* Scientist |
| *Researcher Identifier (ERA commons):* | **ejb761** |
| *Nearest person month worked:* | *1.0* |
| *Contribution to Project:* | *He was involved in optimizing αSyn purification and RT-QuIC assays. He also assisted the PI in preparing the annual report.* |

**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.*

| None |
|---|

## 8. SPECIAL REPORTING REQUIREMENTS

**COLLABORATIVE AWARDS:** *N/A*

**QUAD CHARTS:** *N/A*

## 9. APPENDICES: *N/A*