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TITLE: Interactions of Gut Microbiome, Genetic Susceptibility, and Environmental Factors in Parkinson's Disease

PRINCIPAL INVESTIGATOR: David Standaert, MD, PhD

CONTRACTING ORGANIZATION: University of Alabama, Birmingham, AL

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David Standaert, MD, PhD

EMail: dstandaert@uabmc.edu

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University of Alabama at
Birmingham
UAB
701 S 20th St
Birmingham, AL 35294-0001

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Genetic and environmental factors explain a fraction of Parkinson disease risk, prompting the question if microorganisms in the gut may be the trigger. The main goal of this proposal is to validate and investigate the mechanisms of interaction in a pre-clinical mouse model of PD. To do this, we have created a germ-free (GF) and specific pathogen free (SPF) pre-clinical mouse model in which alpha-synuclein is constitutively expressed under the Thy1 promotor (Thy1-SNCA). We have successfully established stable mouse colonies in both settings and both confirmed the transgene established alpha-synuclein expression via PCR and protein expression in the brain of Thy1-SNCA mice via western blot. In order to test whether pathogenic microorganisms present within the gut trigger PD-like pathology, we have transferred Corynebacteria amycolatum (CA, identified through the work of the Partnering Project) to GF and SPF Thy1-SNCA mice. We have established cultured conditions for CA, a PCR method to detect it, and performed transfers to both germ free and specific pathogen free mice. We have shown that in GF mice, this produces stable colonization. In the SPF mice, however, CA colonization does not occur. During the last research period, we have bred a colony of GF Thy1-SNCA transgenic mice sufficient to test the study outcomes and collected tissue samples for both one month and six month endpoints. Remarkably, we have found that the presence of CA in fact attenuates, rather than enhances, PD-like pathology relative to the gnotobiotic state.

15. SUBJECT TERMS

Parkinson Disease, microbiome, mouse models, alpha-synuclein, gnotobiotic

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1. INTRODUCTION:

Genome-wide studies have identified 28 independent genetic risk variants for idiopathic Parkinson Disease (PD), but each has a small effect, and together, they account for a fraction of the genetic component in PD. Epidemiological studies have associated several elements with PD, most notably, exposure to herbicides/pesticides with increased risk, and cigarette smoking and caffeinated coffee consumption with reduced risk of PD, but as with genetic factors, individual effects are small. Many groups have tested interaction between the genetic and environmental risk factors of PD in hypotheses-driven studies, and we have conducted hypothesis-free genome-wide geneenvironment interaction studies. These studies discovered modifier genes with critical clues to PD pathogenesis, but they were unable to produce any gene-environment interactions that could explain PD risk completely. There must be more to gene-environment interaction in PD than the human genome and the environmental factors that we know of. This research program, awarded under the Partnering PI option, is based on preliminary data that indicates the gut microbiome is the missing link. Dr. Payami is the Initiating PI and will provide a separate progress report. This component of the project, led by the coordinating PI Dr. Standaert, encompasses Aim 5 of the overall proposal: To investigate the mechanisms of interactions. Hypothesis: Corynebacterium will increase alpha-synuclein and accelerate parkinsonian phenotypes in a mouse model. Rationale: Transplant of a PD microbiome has been shown to enhance parkinsonian phenotypes in alpha-synuclein over-expressing mice, but the responsible microorganisms are unknown. Research strategy: We will use Thy1-SNCA specific pathogen-free and germ-free mice and test the effect of introducing Corynebacterium on alpha-synuclein and relevant phenotypes.

2. KEYWORDS

Parkinson Disease, microbiome, mouse models, alpha-synuclein, gnotobiotic

3. ACCOMPLISHMENTS

What were the major goals of the project? The goals of this Partnering PI project are as described in the original Statement of Work, and are listed in the Table below.

Major Goals	**Timeline in months**	**% Complete**
Aim 5. Investigate the mechanisms of interactions in model organisms.		
Major Task 1: Re-deriving Thy1-SNCA mouse in gnotobiotic facility and breeding of SPF mice		
Subtask 1: Re-derive Thy1-SNCA mouse in gnotobiotic facility	1-12	100%
Subtask 2: Breeding of GF Thy1-SNCA mouse in gnotobiotic facility	6-24	100%
Subtask 3: Breeding of SPF Thy1-SNCA mouse in conventional housing facility	1-24	100%
Major Task 2: Transfer of Corynebacterium to SPF and GF mice for 1 month time point		
Subtask 1: Transfer of Corynebacterium to SPF and GFP mice	12-24	100%
Subtask 2: Confirmation of stable Corynebacterium infection by fecal PCR	12-24	100%
Subtask 3: Collection of tissues for 1 month analysis	24-36	100%
Subtask 4: 1 month data collection/analysis	24-36	100%
Major Task 3: Transfer of Corynebacterium to SPF and GF mice for 6 month time point		
Subtask 1: Transfer of Corynebacterium to SPF and GFP mice	24-36	100%
Subtask 2: Collection of tissues for 6 month analysis	36-48	100%
Subtask 3: 6 month data collection/analysis	36-48	100%
Milestone #6: Co-author manuscript on Corynebacterium in Thy1-SNCA mouse model of PD	48	80%

What was accomplished under these goals?

During this four year reporting and extension period, we have:

1. Successfully executed a Materials Transfer Agreement with the University of California, San Diego (UCSD), the source of the mouse line required for these studies.
2. Obtained Thy1-aSyn (SNCA) transgenic mice from UCSD. A total of 5 female mice were received.
3. Established a genotyping protocol to identify the transgenic animals. With the collaboration of UCSD investigators, we purchased appropriate DNA primers and established a PCR assay to detect the presence of the transgene (Figure 1, below).

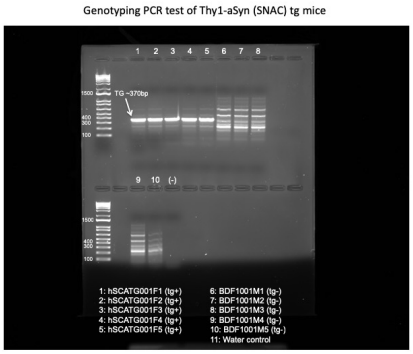

Figure 1. PCR assay for presence of the alpha-synuclein transgene. Lanes 1-5 contain DNA from the transgenic mice provide by UCLA, while 6-10 contain DNA from control mice. The presence of the transgene is indicated by the band at 370 bp.

4. Established breeding of the transgenic animals in a standard mouse housing facility.
5. In order to validate the mouse line, we designed two pairs of PCR primers spanning the transgene (Figure 2) and sequenced the PCR products covering the whole hSNCA transgene and neighboring regions (Figure 3).

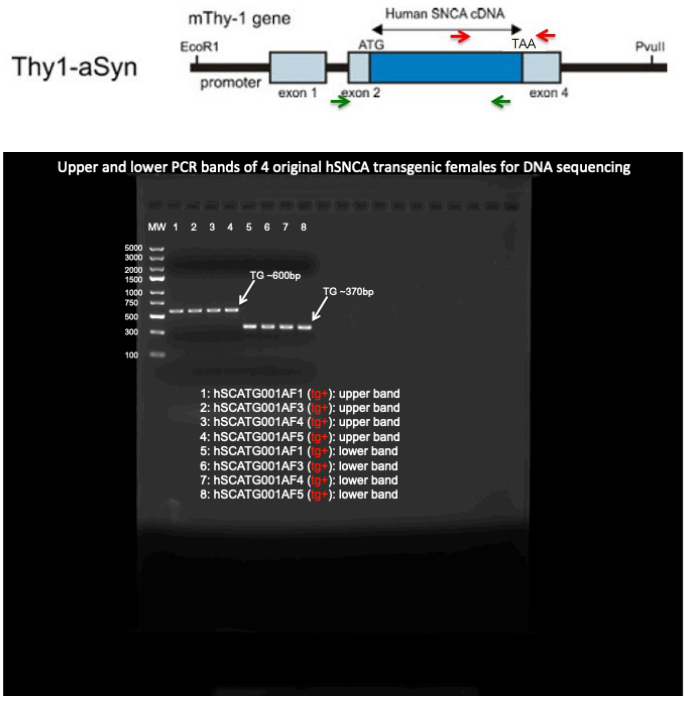

Figure 2. Validation that the hSNCA transgene sequence of the original female breeders is correct. Top illustrates design of a pair of primers specific to an upper region of hSNCA transgene. Lower panel illustrates PCR amplification of upper and lower PCR fragments of hSNCA transgene.

We confirmed that the transgenes of original 4 breeder females are of the correct sequence, an important authentication of the animals.

Figure 3. Sequence alignment of 4 original female breeders by Sanger DNA sequencing with SNCA reference sequence. This demonstrates that the transgene in each animal matches the human SNCA reference sequence.

6. Established breeding of the transgenic animals in a standard mouse housing facility. We have successfully obtained twenty-seven litters of pups derived from our five female founders. Of the pups genotyped so far, 55 are transgene positive. These animals were aged for up to 18 months and their brains at different time points (6, 12, and 18 month) and stools were collected as control materials for germ free TG mouse tissues.

7. We have successfully completed an alpha-synuclein selective western blot to confirm transgene expression in the cortex (CTX), ventral midbrain (MB), and cerebellum (CB) in the transgenic positive animals (Figure 4). Anti-vinculin was blotted as a loading control.

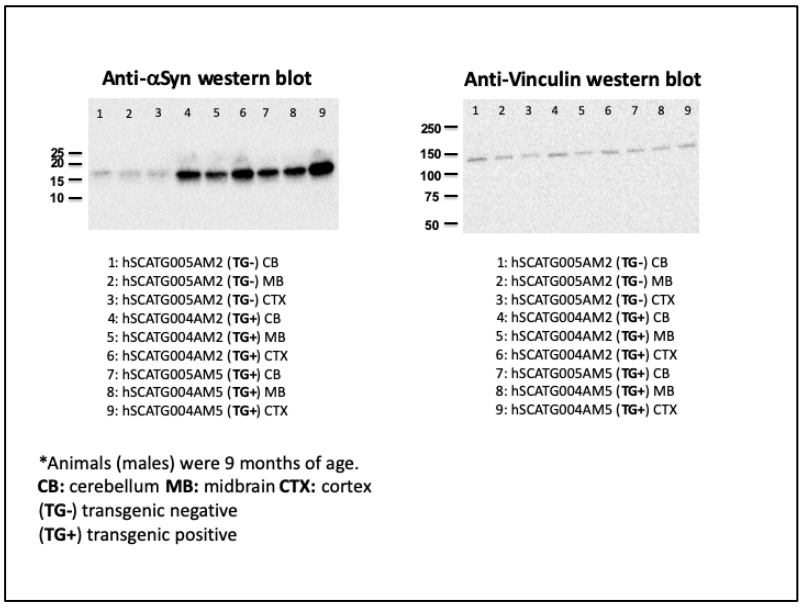

Figure 4: Western blot detecting the overexpression of alpha-synuclein in the cerebellum, ventral midbrain, and cortex of 9 month old transgenic positive (TG+) animals. Anti-vinculin was blotted in the same samples as a loading control.

8. We initially obtained 3 transgenic germ-free females and 9 non-transgenic control male breeders by germ-free mouse derivation, confirmed their germ-free status, and have expanded the germ-free mouse colonies (Figure 5). In addition to GF mouse, specific pathogen free mouse colony has been continously maintained. Both remain fostered within the facility.

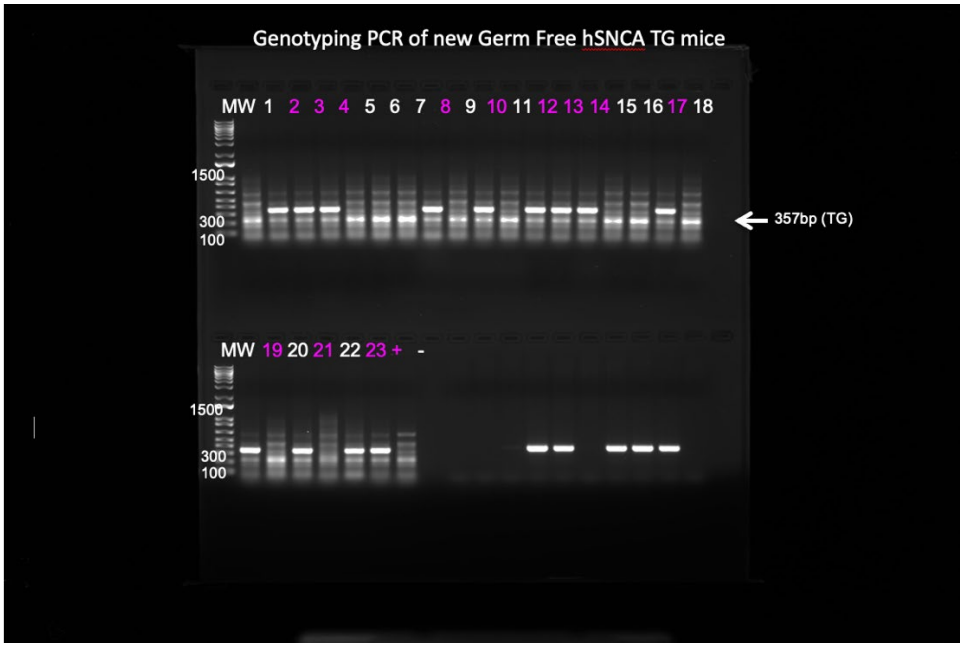

Figure 5. Genotyping PCR results of pups bred from germ-free hSNCA transgenic breeders.

9. Fecal transfer and colonization of target bacterium: We have chosen *Corynebacterium amycolatum* as the specific species to transfer. This is based on work by Dr. Payami in the Partnering Project (NPJ Parkinsons Dis 2020 Jun 12;6:11. doi: 10.1038/s41531-020-0112-6). The bacterium stock was obtained from ATCC, and established in culture. We developed a PCR method to test for the presence of this specific bacterium, as illustrated below. We have tested the colonization of *C. amycolatum* into GF mice by fecal transfer (by naturally feeding stools). They were stably colonized into guts of GF mice for long period of time (up to 6 months) (data is not shown here). Interestingly, the bacteria transferred well to the gut of GF mice, but it did not colonize in that of SPF mice, probably due to the competition with pre-existing gut microbiota (Figure 6).

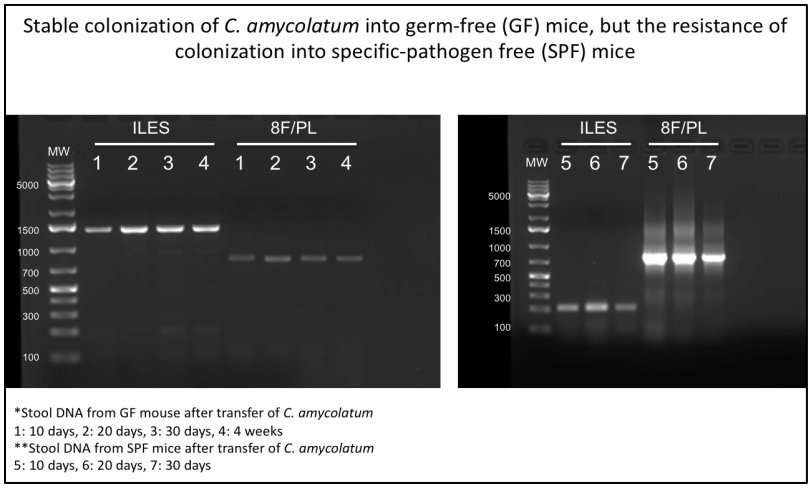

Figure 6. Stable colonization of *C. amycolatum* into germ-free mice, but not into SPF mice

10. During year 4, we generated a large number of experimental TG males by large breeding set-ups (9-10 breeding pairs). As a result, we have produced 49 GF TG males and more TG males for back-up. Of these mice, we transferred *Corynebacterium amycolatum* to 15 germ-free transgenic mice along with 11 cohort mice without bacteria for our 6 month experiment.

11. On the 6 month germ free cohort, we have performed behavioral testing including pole test and open field prior to processing tissues to determine whether germ free conditions change behavioral outcomes. We have confirmed alpha-synuclein specific changes in behavioral phenotypes (Figure 7) and are currently analyzing whether *Corynebacterium amycolatum* altered the behavioral phenotypes.

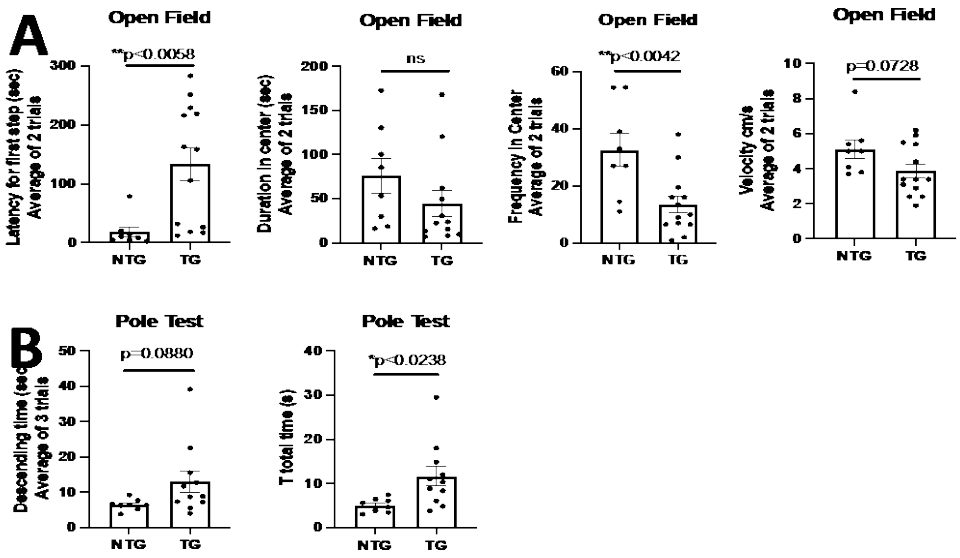

Figure 7: Open field and Pole test results in Germ free NTG vs TG Thy1 mice. TG mice displayed slower initiation of movement and higher anxiety phenotypes in the open field test. Additionally, they displayed significant deficits in pole test including T turn time (time to turn around).

12. One month and 6 months post transfer, we have processed the cohorts, cryoprotected, sectioned and performed immunohistochemistry for IBA-1 (microglial marker) and GFAP (astrocyte marker). We have performed microscopy and analysis in the hippocampus, cortex, striatum, and ventral midbrain to determine whether *Corynebacterium amycolatum* transfer exacerbates micro and astrogliosis.
13. At 6 month time point, we have found in the behavioral tests that GF TG mice colonized with *Corynebacterium amycolatum* (CA) have better motor skills and less anxiety phenotypes than cohort gnotobiotic mice without CA (Figure 8), suggesting a protective role of CA in PD development relative to the gnotobiotic state.
14. From immunohistochemistry of GF mouse brain we found that pSer129 expression is reduced in GF TG mice w/CA compared to GF TG mice w/o CA in several brain regions including striatum, hippocampus and thalamus at 6 month time (Figure 9). There is no difference at 1 month time (data not shown). This result

correlates with the findings of behavioral tests and supports the idea that *Corynebacterium amycolatum* may reduce aSyn toxicity compared to the gnotobiotic state.

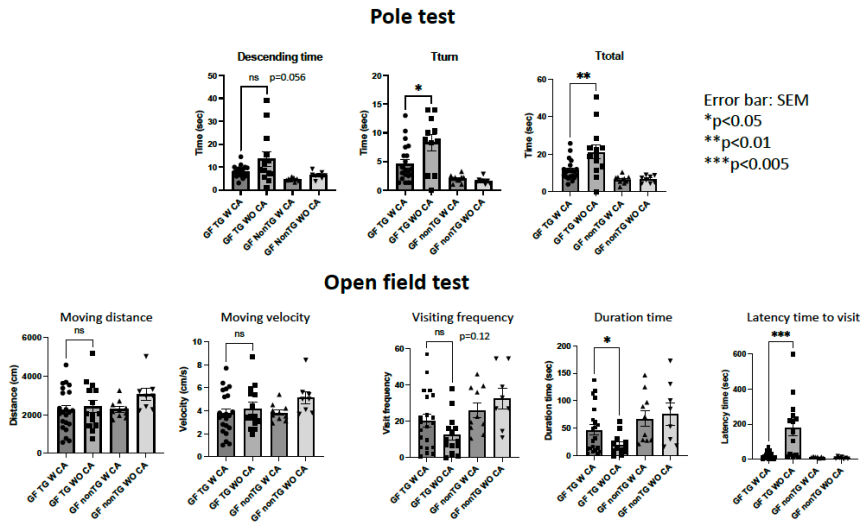

Figure 8: Behavioral tests: Open field and pole test results in Germ free TG mice w/ vs w/o *Corynebacterium amycolatum* (CA). For internal controls, GF Non-TG mice were compared together. Latency time to visit defines the time when the mouse visits first time from the corner to the inner zone of open box.

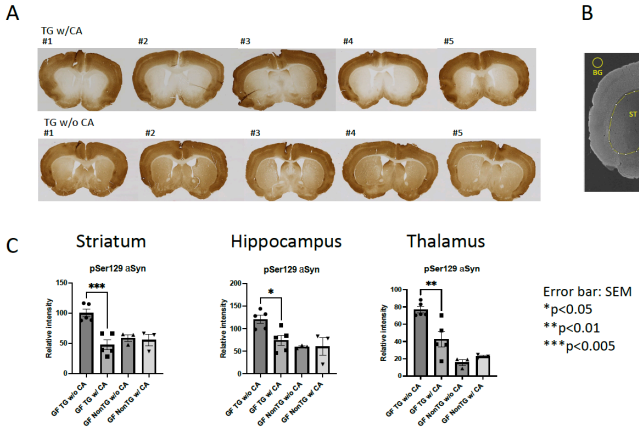

Figure 9: Inhibition of pSer129 aSyn aggregate formation in GF TG mice by *Corynebacterium amycolatum* (CA). (A) GF mouse brain sections w/ and w/o CA were stained with anti-pSer129 aSyn antibody and developed with DAB showing drastic reduction of pSer129 aSyn in only TG mice w/CA, (B) Quantification of pSer129 aSyn level of GF mouse striatum. Relative expression level (intensity) is calculated by mean gray value (MGV) of left-side striatum subtracted from background MGV, (C) pSer129 aSyn levels in different brain regions were compared by the same quantification method. Striatum, hippocampus, and thalamus of GF mice w/CA show statistically significant differences from a control TG mouse group w/o CA.

15. During grant extension period to the final reporting time, we achieved the following result by histology analysis. At 1 month time point, we have found that compared to TG mouse cohort w/o *Corynebacterium amycolatum* (CA), GF TG mice colonized with CA show reduced neuroinflammation states of microglia such as dampening microglia compartment expansion and reduced numbers of $MHCII^{+}$ microglia as an indicator of microglia activation (Figure 10). This data shows that at the early time point CA is associated with less microglia activation than the gnotobiotic state, slower α-Syn-mediated PD pathology, and less severe behavioral deficits at the late stage.

16. The overall conclusion is contrary to our original hypothesis that CA would accelerate α-Syn-mediated PD pathology in this model system. Instead, the presence of CA seems to retard α-Syn-mediated PD pathology relative to the fully gnotobiotic state. We expect this result will be of substantial interest to the scientific community, and will prompt future exploration of the role of CA and other microbiome organisms in α-Syn-mediated PD pathology.
17. These results have been presented in abstract form, and a manuscript describing the findings is nearing completion.

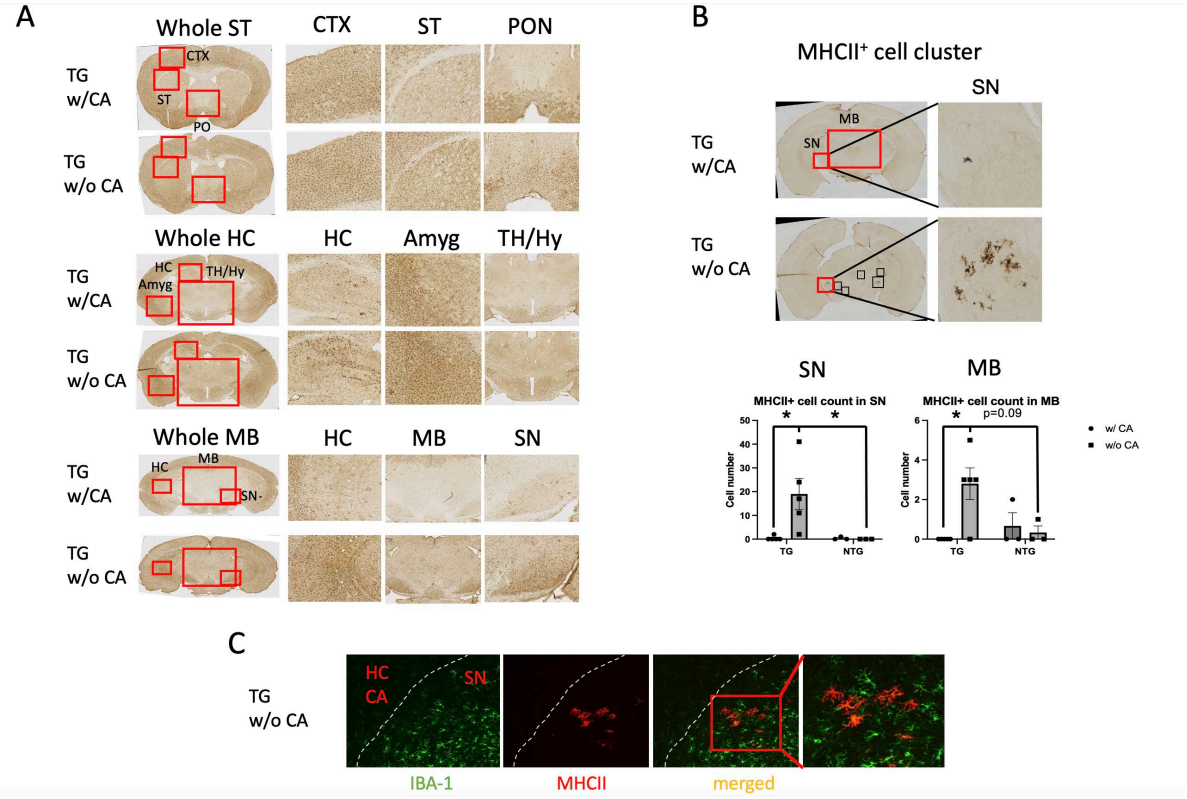

Figure 10: *Corynebacterium amycolatum* reduces activation and spreading of microglia and MHCII induction of MG cells during early PD development relative to the gnotobiotic state. (A) Reduced density of microglia in the thalamus and other rgions of TG mice w/ CA relative to TG mice without CA. Microglial cells were examined by staining with anti-IBA-1 Ab (microglia marker) in the brain. The distribution and density of microglia were compared between TG mice w/ CA and w/o CA in various brain regions (the whole ST (CTX (cortex), ST (striatum), PON (pre-optic nucleus), whole HC (HC (hippocamps), Amyg (Amygdala), TH/HY (thalamus/hypothalamus), and whole MB (MB (midbrain), SN (substantia nigra)) regions) at the early time point (1 month after CA transfer). The pictures only shows one representative from each mouse group. (B) Decreased MHCII$^+$ cell number in SN and MB area of TG mice w/ CA. MB sections of mouse brains were stained with anti-MHCII mb and MHCII expression in the cells of the sub-brain regions were compared between TG mice w/CA and w/o CA. The pictures only shows one representative from each mouse group. Statistical analysis was done by student t test (TG w/ CA (N=5), TG w/o CA (N=5), Non-TG w/CA (N=3) and Non-TG w/o CA (N=3)). Error bar shows SEM (standard error of the mean) (*p<0.05, **p<0.01, ***p<0.005). (C) Identification of MHCII$^+$ cells to be microglial cells. To identify the MHCII$^+$ cells, the MB sections were doubly stained with anti-MHCII and anti-IBA-1 antibodies and the identity was confirmed the co-staining of two markers. The pictures only shows one representative from TG mice w/o CA. All brain sections were examined in an unbiased manner by an investigator blinded to the treatment groups.

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

- *We have recruited and trained an undergraduate, Nikhita Mudium, to assist with microscopy and image analysis.*

How were the results disseminated to communities of interest?

- *The results have been presented in abstract form and a poster at the 2023 SIS (Southeastern Immunology Symposium, Nashville, TN, 6/13/2023)*
- *A manuscript describing the results is in late stages of preparation and will be submitted shortly.*

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

This is the final report.

4. **IMPACT:**
   - **What was the impact on the development of the principal discipline(s) of the project?**
     - *In this project, we explored the role of colonization of the gut microbiome with CA on α-Syn-mediated PD pathology. Our original hypothesis was that this would accelerate α-Syn-mediated PD pathology. We found, to our surprise, that the presence of CA in fact reduces the severity and consequences of α-Syn-mediated PD pathology relative to the germ-free, gnotobiotic state. This result will require a re-assessment of the role of gut micro-organisms in α-Syn-mediated PD pathology, and suggests that the effects of a specific organism such as CA may depend on the background in which it resides (e.g., normal flora vs. gnotobiotic state). We expect that this will draw considerable interest from the field and will lead to further exploration of the role of the gut microbiome in α-Syn-mediated PD pathology.*
   - **What was the impact on other disciplines?**
     - *These results are also informative for the broader field studying the role of the microbiome in systemic inflammation and neurodegenerative disorders.*
   - **What was the impact on technology transfer?**
     - *Nothing to Report*
   - **What was the impact on society beyond science and technology?**
     - *This work demonstrates clearly that the gut microbiome has a powerful effect on brain disorders. With further development, this may lead to new approaches for preventing or treating Parkinson disease and other serious brain disorders.*
5. **CHANGES/PROBLEMS:**
   - **Changes in approach and reasons for change**
     - *The project has proceeded largely as planned..*
   - **Actual or anticipated problems or delays and actions or plans to resolve them**
     - *There have been some minor delays due to the impact of COVID-19, estimated at about 3 months. Overall, these have not delayed the project greatly. All experimental work is complete, and only the final steps in manuscript submission remain.*
   - **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*

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  - The *work has been presented in 2023 SIS (Southeastern Immunology Symposium, Nashville, TN, 6/13/2023) in a form of poster.*
  - *A manuscript describing the results is in the late stages of preparation, and will be provided when complete.*
- **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

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: David Standaert, MD, PhD
Project Role: Principal Investigator
Researcher Identifier (e.g. ORCID ID): 0000-0003-2921-8348
Contribution to Project: Dr. Standaert oversaw the project start up, and has conducted ongoing meetings with project personnel to design, implement, and troubleshoot experiments.

Name: Ashley Harms, PhD
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): 0000-0002-7054-2812
Contribution to Project: Dr. Harms managed the day to day operation of the project and supervised the Research Associate assigned to this project.

Name: Woong-Jai Won
Project Role: Research Associate
Researcher Identifier (e.g. ORCID ID): N/A
Contribution to Project: Dr. Won has been responsible for monitoring animal care and breeding, collecting tail DNA, extracting DNA, developing and performing the genotyping assay.

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

8. **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:**
  - This report encompasses reporting for the Collaborating PI of the this award. The Initiating PI, Dr. Haydeh Payami, will submit a separate report.

9. APPENDICES:

- There are no appendix materials.