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Award Number: W81XWH-18-1-0233

TITLE: Harnessing Neuroplasticity Genes to Combat Synucleinopathy-Mediated Axonopathy

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REPORT DATE: June 2019

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE *June 2019*	**2. REPORT TYPE** Annual	**3. DATES COVERED** *(From - To)* 1 Jun 2018 –31 May 2019

4. TITLE AND SUBTITLE

Harnessing Neuroplasticity Genes to Combat Synucleinopathy-Mediated Axonopathy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-18-1-0233

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

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

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

10. SPONSOR/MONITOR'S ACRONYM(S)

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

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The earliest stages of synucleinopathy have been difficult to study due to the fact that most animal models of Parkinson's disease (PD) fail to recapitulate the progression of synucleinopathy to neurodegeneration. The alpha-synuclein (α-syn) preformed fibril (PFF) synucleinopathy model exhibits a distinct stage of accumulation of α-syn inclusions in tyrosine hydroxylase immunoreactive (THir) neurons in the substantia nigra pars compacta (SNpc) months prior to the ultimate degeneration of the nigrostriatal system. In the context of the early phases of synucleinopathy in the α-syn PFF model, laser capture microdissection was used to collect phosphorylated α-syn (pSyn) immunoreactive SNpc neurons in PFF-injected rats and SNpc THir neurons in control-injected rats. RNA was isolated and RNASeq used to identify gene expression changes between SNpc neurons with and without pSyn inclusions. Results from male rats have identified 102 candidate genes (24 expressed only with PFF treatment, and 78 only in control animals). The known functions of these genes are being identified and those involved in neuroplasticity and confirmed with digital droplet PCR will be manipulated with designed adeno-associated viruses in the next phase with the goal to mitigate neurodegeneration.

15. SUBJECT TERMS
Synuclein, Synucleinopathy, neurodegeneration, RNASeq

16. SECURITY CLASSIFICATION OF: U			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	7	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)

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Introduction

Parkinson's Disease (PD) is the second most common neurodegenerative disease. Hallmarks of PD include motor impairment, progressive degeneration of the nigrostriatial dopamine (DA) neurons, and the accumulation of Lewy Bodies (LBs), or cellular inclusions containing alpha-synuclein (a-syn). The first genetic links to familial PD were mutations and copy number variants in the a-syn gene (*snca*) [1-4]. Though heredity is a factor in ~5-10% of total cases, the overall the etiology of sporadic PD is unknown. In regards to neurodegeneration, the loss of axons or axonopathy has been shown in animal models, as well as patients, to occur prior to the loss of cell bodies of the nigrostriatal neurons [5]. Furthermore, there is abundant evidence to support the role in early PD of impaired axonal guidance and transport, degeneration of synapses, as well as dysregulation of genes encoding proteins known to interact with a-syn in the synapse [6]. Ultimately, the pathophysiological mechanisms in PD appear to culminate in overt axonopathy, with near complete denervation of the caudate putamen within four years of PD diagnosis, preceding degeneration of nigral soma [7].

In the a-syn PFF-seeded synucleinopathy model, exogenous a-syn PFFs injected into the striatum are taken up into nigrostriatal terminals, leading to the templating, hyper-phosphorylation, accumulation, and subsequent formation of endogenous a-syn oligomers and fibrils (Figure 1A). In the both the terminals and cell bodies of the substantia nigra pars compacta (SNpc) there is accumulation of inclusions that are positive for phosphorylated a-syn (Figure 1B), Thioflavin-T and ubiquitin as well as proteinase-K resistant. In addition to the SNpc, inclusions are also present in the cortex, thalamus, and amygdala; regions with neurons that also innervate the striatum [8]. An overview of the time course of events in the a-syn PFF-induced synucleinopathy cascade in the rat nigrostriatal system is illustrated in Figure 1C. Specifically, a-syn hyperphosphorylated inclusions (gray line) form in the SNpc as early as one month, and peak around 1-2 months post-injection. At this same early time point a significant increase in tyrosine hydroxylase (TH) immunoreactivity is observed in the striatum (red line, Figure 1C, [9]), suggesting compensatory changes in the axon terminals in response to accumulating inclusions. This response may be linked to differential gene

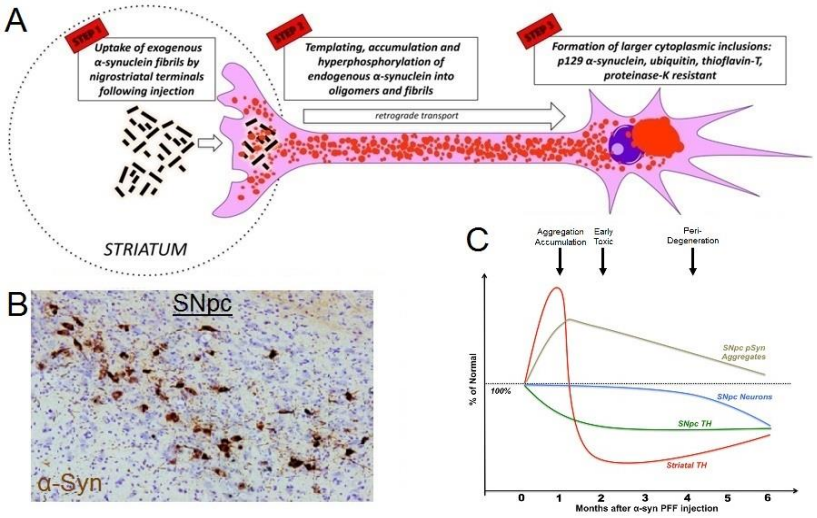

Figure 1. The a-syn PFF-seeded synucleinopathy model. ***A.*** *Diagram showing the uptake a-syn PFFs in the striatum and subsequent formation of inclusions in the SNpc.* ***B.*** *Representative image of inclusion containing SNpc neurons at one month post-injection.* ***C.*** *Time-course of inclusion formation, TH expression and neurodegeneration.*

expression of neuroplasticity genes. At two months, nigral terminals and cell bodies begin to lose their dopaminergic phenotype as evidenced by loss of TH (green line, Figure 1C). Neuronal loss in the SNpc (cell death rather than earlier loss of phenotype) however, does not occur until months 4-6 post-injection (blue line) associated with a >50% reduction in striatal DA tissue content. Using our current optimized parameters we can achieve 50% SNpc degeneration at this time point [9]. Further, loss of SNpc neurons occurs bilaterally despite the fact a-syn that inclusions are observed only within the ipsilateral SNpc [9]. Thus, the nigral synucleinopathy induced by a-syn PFF injection reveals at least three distinct stages:

1) *Month 1: Aggregation and Accumulation Phase*:
   Inclusions are newly formed/forming, neurons and terminals attempt to compensate.
2) *Month 2: Early Toxic Phase:* Decrease in TH protein in both the soma and terminals.
3) *Month 4: Peri-Degeneration Phase:* Nigral neuron death initiates and continues.

The a-syn PFF model presents a unique opportunity to examine gene expression changes that occur throughout the progression of the dynamic phases of synucleinopathy, specifically during aggregate accumulation and early toxic phases. In the PD brain, pathology of nigrostriatal terminals is likely to be the earliest consequence of synucleinopathy in the SNpc [7].

The project seeks to use laser capture microdissection (LCM) to specifically isolate DA neurons in the SNpc, followed by RNA sequencing (RNASeq) to identify genes associated with neuroplasticity altered in early phases of synucleinopathy in male and female rats. Candidate genes from the RNASeq results can then be selected and manipulated with adeno-associated viruses (AAVs) designed to increase or knockdown expression of the candidate gene. Manipulation of these candidate genes will then be tested in the context of the PFF model to determine if synucleinopathy has been mitigated.

Body

The project is progressing along, but is behind schedule due to unexpected issues out of our control, as well as difficulties associated with the technique. In the beginning, there was a delayed start due to the financial processing associated with the university, which delayed access to the account the funds were transferred to until the end of July. We were able to quickly work out our rapid staining technique to identify the cells of interest for LCM and performed the surgeries for the RNASeq experiment. Unfortunately, after surgeries were performed, we were contacted by a representative from Charles Rivers Laboratories, where we purchase our rats, about a possible issue with the genotypes of the rats. An issue occurred with their breeding pairs that resulted in rats with an inconsistent genotype. Since this could cause problems, Charles Rivers Laboratories offered to genotype samples taken from the animals. From the 48 animals ordered, 36 animals had variable genotypes and could not be used. As these were spread randomly and impacted all treatment groups, the entire experiment needed to be repeated.

To solve this issue, Charles Rivers Laboratories was contacted and has refunded the rat purchase, and compensated the lab for rat housing, surgical supplies, and time lost.

Charles Rivers Laboratories, when asked, has provided me a letter documenting their breeding issue (provided in the quad report, but can be resent on request). As not to further delay the project and because it was predicted to be months before wild-type rats of that background would be available, new rats from a separate vendor (Envigo) were ordered, surgeries prioritized, and animals were ready to process 3-4 months behind schedule.

Animals were processed, and LCM used to isolate cells. To maintain RNA integrity, the tissue also had to be processed immediately after sectioning. This slowed the processing of the tissue and delayed the RNASeq. The RNA yield was lower than expected (~1-2 ng) and preamplification was required post-rRNA depletion and prior to library preparation for RNASeq. As to not further delay the project, the male samples were sent for RNASeq and downstream analysis first, with the female samples to be sent after the analysis of the male samples. The initial results from the males has recently been received and genes implicated still needs to be identified based off of their ensemble numbers. There are 24 genes expressed only in the animals that received the PFFs (both at 1 and 2 months) and 78 that are only expressed in the control animals. While the female samples are being processed, these 102 genes and their functions will be identified, and results confirmed with digital droplet PCR with the remaining tissue. Once confirmed, construction and validation of AAVs can begin for the next phase of the project.

Key Research Accomplishments

- Lobbied Congress with other researchers in conjunction with the Michael J Fox Foundation for the increase and continuation DOD CDMRP and NIH funding (Researchers on the Hill Day).

Reportable Outcomes

- Poster presentation at the 2019 World Parkinson's Congress (Kyoto, Japan)
- Selected for the poster tour at the 2019 World Parkinson's Congress (Kyoto, Japan)
- Abstract (poster) accepted for 2019 Society for Neuroscience meeting (Chicago, Il)
- Abstract submitted for 2019 Grand Challenges in Parkinson's Disease (Grand Rapids, MI)

Conclusions

The project has led to the identification of a rapid staining technique for cells for LCM, that will be useful in the future to our and other labs. The RNASeq results from the male rats has identified 102 potential candidate genes that need to be identified based on their Ensemble reference number and categorized based on predicted function. We are currently using the male results to better prepare the female samples for RNASeq and should have those results shortly.

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Appendices

None