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TITLE: Airborne pollutants as triggers of Parkinson’s disease via the olfactory system

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14. ABSTRACT

We are interested in determining whether ambient air pollutants impact the development of Parkinson’s disease by increasing α-synuclein (α-syn) pathology via inflammation. After completing Specific Aim 1, wherein we found activation of microglia relative to controls in contralateral olfactory structures, we exposed mice to nano-particulate matter (nPM) both before and after α-syn pre-formed fibril (PFF) injection into the right olfactory bulb. We have completed postmortem densitometry for spread of α-syn through olfactory structures and stereology for Lewy body presence and neuron counts in the anterior olfactory nucleus but found no differences between groups in these analyses. We are continuing optimization of inflammatory marker antibodies for immunofluorescent colocalization analyses of nPM- and PFF-related neuroinflammation.

15. SUBJECT TERMS

Pre-formed fibrils (PFFs), α-synuclein (α-syn), phosphorylated serine 129 (pSer129), nano-particulate matter (nPM), neuroinflammation, Parkinson’s disease (PD)

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1. INTRODUCTION

This project is entitled "*Airborne pollutants as triggers of Parkinson's disease via the olfactory system"* and has two arms: (A) Define the effects of exposure to nano-sized particle matter (nPM) on the development and progression of α-synucleinopathy in olfactory structures by combining two experimental paradigms and the pre-clinical testing of two drugs (ibuprofen and MDSC-0160). (B) Examine the role of ambient air pollutants in olfactory impairment among older adults in order to understand early stages of PD development. The goal of this multidisciplinary project is to improve our understanding of the early stages of PD development by defining the influence of air pollutants on the development and progression of α-synuclein pathology *in vivo*, and on olfactory dysfunction among older adults. We will pursue experimental (Aims 1-4) and epidemiological (Aims 5-7) studies addressing common research questions. The second year of this project at the Van Andel Research Institute saw the completion of Aim 1 analyses, the commencement of Aim 2 animal experiments, and subsequently the extraction, processing, and histological and stereological analysis of olfactory tissues.

2. KEYWORDS

Pre-formed fibrils (PFFs), α-synuclein (α-syn), phosphorylated serine 129 (pSer129), nano-particulate matter (nPM), neuroinflammation, Parkinson's disease (PD)

3. ACCOMPLISHMENTS:

Major Goals of the Project (from approved SOW):

<u>SPECIFIC AIM 1:</u> Determine the effects of exposing mice to nPM after triggering of PFF pathology (Months 6-16)

1. Inject C57BL/6NJ mice (n=96) with PFFs.

   Validation experiment accomplished (n=32), 10/12/17 (Y1Q1)

   Specific Aim 1 injections (n=64) accomplished 1/18/18 (Y1Q2)

2. Expose C57BL/6NJ mice to nPM.

   Validation experiment accomplished (n=32), 11/09/17 (Y1Q1)

   Specific Aim 1 exposure (n=64) accomplished 2/14/18 (Y1Q2)

<u>Milestones in this reporting period:</u>

1. Histological analysis of Iba-1 for Specific Aim 1 full experiment **completed 12/10/18 (Y2Q1)**

SPECIFIC AIM 2: Determine the effects of exposing mice to nPM before triggering of PFF pathology (Months 12-30)

1. Pre-expose mice to nPM for 3 weeks before injections

Specific Aim 2 pre-stereotactic injection exposure (n=64) initiated 10/8/18, accomplished 10/28/18 (Y2Q1)

2. Inject mice with PFFs (as in Aim 1).

Specific Aim 2 injections (n=64) initiated 10/29/18, accomplished 11/1/18 (Y2Q1)

3. Post-expose mice to nPM for 7 weeks after injections

Specific Aim 2 post-stereotactic injection exposure (n = 64) initiated 11/2/18, accomplished 12/20/18 (Y2Q2)

Milestones in this reporting period:

1. Complete collection and delivery of brains to VARI **collection completed 12/21/18; brains received 1/15/19 (Y2Q2).**
2. Biochemical analyses **completed at USC (Y2Q4).**
3. Histological analyses (starts after 1 month for sectioning):
    a. pSer129 ImageJ densitometry analysis **completed 6/12/19 (Y2Q3)**.
    b. NeuN stereology analysis completed **9/4/19 (Y2Q4)**.
    c. pSer129 pathology scoring analysis **completed 9/13/19 (Y2Q4)**.
    d. Iba-1 and inflammatory cytokine immunofluorescence double stain optimization **ongoing**.
4. Data analysis, manuscript preparation, and submission **ongoing.**

Specific Aims 3 & 4: **not yet initiated**

What was accomplished under these goals?

1) *Major activities:*
    a. Histological analysis of microglia (Iba-1) in Aim 1 brains (by VARI personnel) (Y2Q1).
    b. Pre-stereotactic injection exposure of Aim 2 C57BL/6NJ mice to nPM at USC (by USC personnel) (Y2Q1).
    c. Stereotactic injection of Aim 2 C57BL/6NJ mice with PFFs at USC (by VARI personnel) (Y2Q1).
    d. Post-stereotactic injection exposure of Aim 2 C57BL/6NJ mice to nPM at USC (by USC personnel) (Y2Q2).
    e. Collect Aim 2 experiment brains at USC and deliver to VARI (Y2Q2).
    f. Sectioning Aim 2 experiment brains (by VARI personnel) (Y2Q2).

g. Staining, imaging, and densitometry analysis of pathologic α-syn (pSer129) in Aim 2 brains (by VARI personnel) (Y2Q2, Y2Q3).

h. Staining and stereological analysis of neuronal (NeuN) cell counts in Aim 2 brains (by VARI personnel) (Y2Q4).

i. Re-analysis of pathologic α-syn by pSer129 Lewy body stereology (by VARI personnel) (Y2Q4).

j. Optimization of inflammatory cytokine antibodies for neuroinflammation analyses (by VARI personnel) (ongoing).

2) *Specific objectives:* Perform the first collaborative study to examine effects of exposure to nano-sized particle matter (nPM) on the development and progression of α-synucleinopathy in olfactory structures.

3) *Significant results or key outcomes:*

– **Y2Q1:** Specific Aim 1 mice were initially injected with either saline or α-syn PFFs and subsequently exposed to either forced air (air) or nano-particulate matter (nPM) to examine the effects of nPM on PD-like synucleinopathy (previously reported in Year 1 annual report) and neuroinflammation, which is reported here.

A. Specific Aim 1 microglial activation (Iba-1) analyses:

Imaging and analyses for Iba-1 cell counts and morphology (an indicator of activation of the neuroimmune microglia cells) were completed during this reporting period. Relative microglial activation was defined by the ratio of microglia cell area to cell perimeter (morphology analysis). Iba-1 analyses were conducted for the olfactory bulbs (OB), anterior olfactory nucleus (AON), and cortical amygdala (CoAM).

For cell count analysis, we observed a significant increase ($p < 0.05$) in microglia counts in the pooled PFF-injected animals in the right OB (ipsilateral to injection site; **Figure 1**), as compared to saline-injected animals. However, we did not observe any effect of nPM exposure to microglia abundance across the olfactory regions analyzed, nor did we observe an interaction between PFF injection and nPM exposure on microglia numbers. Increases in microglia numbers in PFF-injected animals in the ROB could be explained by migration of microglia in response to insults related to presence of PFFs.

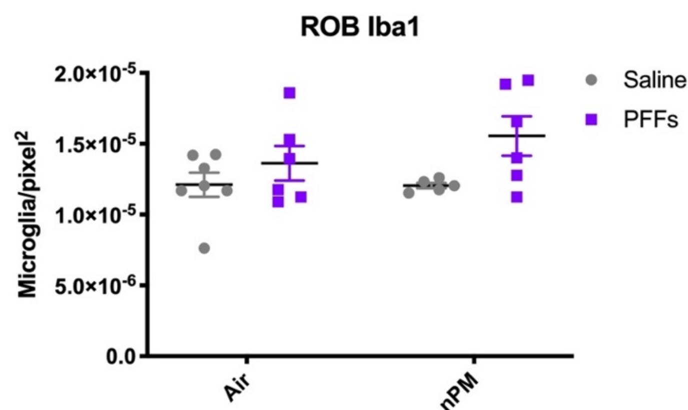


Figure 1: Microglia (Iba-1) cell counts in the right olfactory bulb (ROB) after four weeks of nPM or air exposure. Main effect of pathology (PFF vs. saline injection; $p < 0.05$). Mean ± s.e.m.

For morphology analysis shown below in **Figure 2**, we observed a significant interaction between PFF injection and nPM exposure in the left OB ($p < 0.05$) and left AON ($p < 0.01$; contralateral to injection site) on relative activation of microglia compared to controls. We observed simple main effects of pathology and

exposure in the LAON when comparing microglia from PFF- and saline-injected groups in the nPM-exposed animals, and in comparing microglia from the nPM- to air-exposed groups in the PFF-injected animals ($p < 0.05$). Here, activation of microglia, an indicator of neuroinflammation, is defined as a higher ratio of cell area to perimeter, since activated (or amoeboid) microglia have a larger cell body (increasing area) and retracted processes (reducing perimeter), whereas resting (or ramified) microglia have a smaller cell body and longer processes extending outward from the cell body. However, we did not observe any significant main effects of pathology or exposure, nor interactions between the two, in inducing relative activation of microglia in any other brain regions.

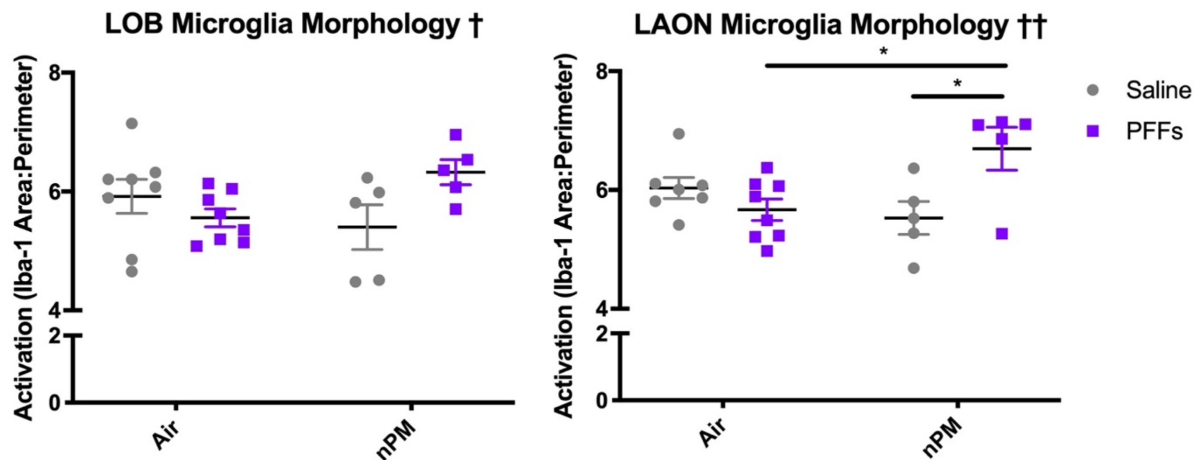


Figure 2: Relative morphology of microglia (Iba-1) in the left olfactory bulb (LOB) and left anterior olfactory nucleus (LAON) after four weeks of nPM or air exposure. Higher area:perimeter ratio is indicative of activated microglia relative to a lower ratio. Significant interaction between pathology and nPM exposure was observed in both brain regions (†: $p < 0.05$; ††: $p < 0.01$), with significant differences observed in the LAON between nPM- and air-exposed animals within the PFF-injected group, and between PFF- and saline-injected animals within the nPM-exposed group (*: $p < 0.05$). Mean ± s.e.m.

B. Specific Aim 2 PFF injections:

From 10/29-11/1/2018, after pre-injection exposure of the mice to nPM or air at USC (three weeks, 10/8/18-10/28/18), Dr. Christopher Tulisiak and Ms. Lindsay Meyerdirk (VARI personnel) traveled to USC to perform PFF or saline injections into 64 C57BL/6NJ mice, with USC personnel. On 11/2/18, these mice continued their respective air or nPM exposures for the seven-week post-injection exposure period.

– Y2Q2: The 64 Aim 2 mice injected with PFFs or saline were exposed to nPM by USC personnel as stated above. After seven weeks of post-PFF injection nPM exposure, the mice were euthanized (week of 12/21/18) and processed for analysis at USC by VARI and USC personnel according to the Statement of Work. VARI staff received 32 brains from USC on 1/15/19. Sectioning of these 32 brains was initiated 1/22/19.

– Y2Q3: Sectioning of the Aim 2 experimental brains was completed 3/22/19.

Specific Aim 2 α-syn (pSer129) densitometry analysis:

Immunostaining for α-syn pSer129 in the Specific Aim 2 experimental brains was completed per Dr. Brundin's laboratory's standard immunohistochemistry protocols by 4/16/19. Imaging of immunostained

brain slices (three olfactory structure regions, bilaterally in both hemispheres, per brain) from 32 brains was completed 5/21/2019. Analyses of α-syn pSer129 immunostaining were conducted using an ImageJ densitometry plugin for quantification of the pixel area surpassing a threshold intensity (α-syn pSer129-stained tissue), and were completed 6/12/2019.

We compared the amounts of α-syn pSer129 immunostaining in PFF- and control saline-injected animals in both the air-exposed control and the nPM-exposed experimental groups. We quantified α-syn pSer129 immunostaining and defined it as stained area multiplied by mean intensity across the whole of the image.

As predicted, and in line with our earlier findings using shorter exposure to nPM in Aim 1, we did not detect any α-syn pSer129 pathology in PBS-injected control animals. This demonstrates that even long-term nPM exposure alone (i.e., in saline-injected controls) is insufficient for the development of α-syn pathology in our experimental paradigm.

To test our hypothesis that prolonged nPM exposure exacerbates α-syn pSer129-positive pathology, we directly compared the immunostaining between the two PFF-injected groups (air- and nPM-exposed) in the OB, AON, and CoAM (**Figure 3**). In summary, after initial densitometry analysis, we observed no significant effects of nPM exposure on α-syn pSer129 pathology. Notably, the ipsi- and contralateral AON and contralateral OB exhibited a non-significant trend towards reduced spread of α-syn in nPM-exposed animals, which is actually counter to our hypothesis. There are several potential explanations for this preliminary result, as explained below.

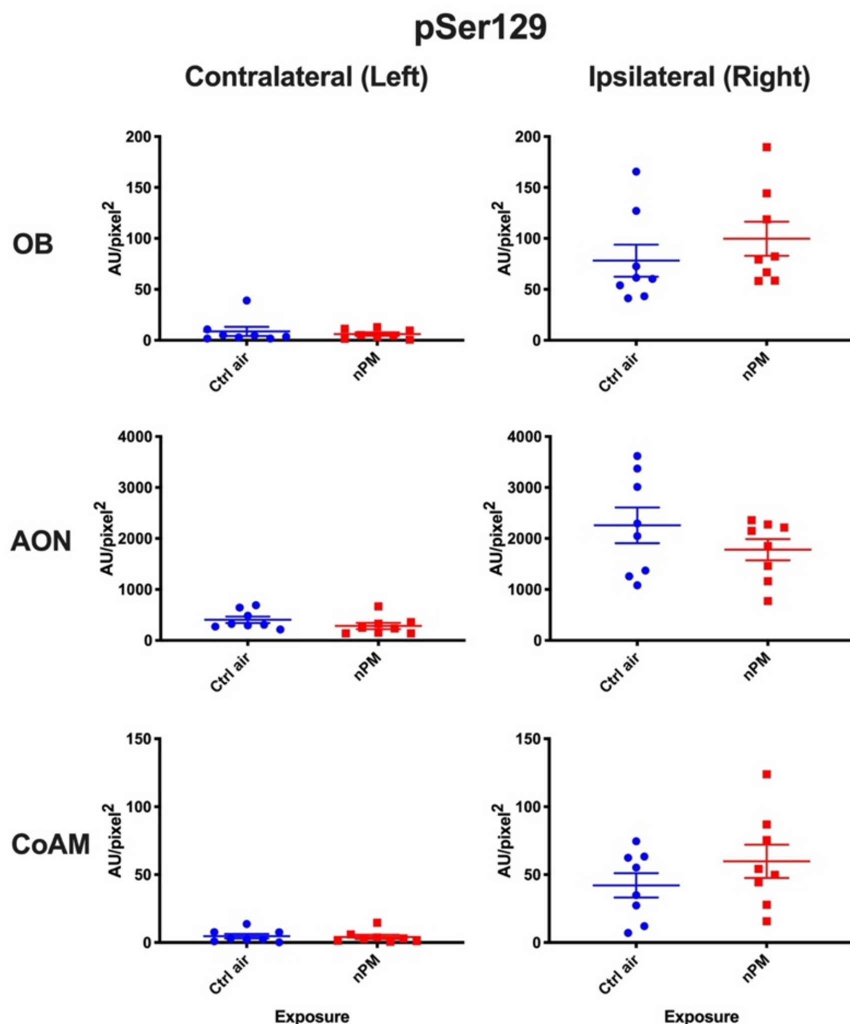


Figure 3: α-Syn aggregate pathology (phosphorylated serine 129 – pSer129) in olfactory areas after ten total weeks (three weeks pre-PFF injection, seven weeks post-PFF injection) of nPM or air (control) exposure. Left: olfactory structures contralateral to PFF injection (which was made in the right olfactory bulb, ROB). Right: olfactory structures ipsilateral to PFF injection in ROB. No statistically significant differences were observed in pSer129 pathology and spread in any brain region analyzed, nor in pooled brain regions (not shown). AU: arbitrary units; Ctrl air: forced air-exposed control group; nPM: nano-particulate matter-exposed group; R: right; L: left; OB: olfactory bulb; AON: anterior olfactory nucleus; CoAM: cortical amygdala. Mean ± s.e.m.

First, it was possible that prolonged nPM exposure (combined with propagation of α-syn pathology) led to death of neurons that contained α-syn aggregates and, as a consequence, there was a reduced amount of α-syn pSer129-immunopositive cells in olfactory structures. We clarified if this is the case (Y2Q4) by counting the number of remaining neurons in olfactory structures, starting with the AON, which we have here and previously shown to experience the greatest load of pSer129 presence compared to the other analyzed regions, using unbiased stereology.

Second, it was possible that prolonged nPM exposure triggers an inflammatory response that promotes the degradation of α-syn, in concert with or separately from the possible neuronal death mentioned above. To determine if this was the case, we will immunostain for the microglia marker Iba-1 and an inflammatory cytokine for analysis of general neuroinflammation using immunofluorescent double staining, which affords the ability to also perform colocalization analyses. This will allow us to confirm that microglia are activated and have expressed neuroinflammatory cytokines, and will also allow us to quantify cytokine expression (o, Y2Q4).

Third, the ImageJ densitometry analysis method we used to make this initial assessment of pSer129 α-syn staining had inherent limitations because analysis parameters needed to be adjusted for staining intensity, leading to misrepresentation of mean staining intensity and ultimately to super-threshold pixels discovered by the ImageJ macro. Since the output is a function of mean staining intensity and total stained area, the analysis is vulnerable to variability in background staining which can occur between mouse brains and between sections from the same brain. Furthermore, densitometry does not distinguish between Lewy bodies and Lewy neurites. While stereological analysis of Lewy bodies is a possibility, that approach does not allow us to count Lewy neurites. Notably, Lewy neurites, a key hallmark of PD pathology, are a significant part of α-syn pathology in these experiments.

More adequate quantification tools are needed to provide a better picture of what is happening in our paradigm. Thus, in this reporting year we have been working diligently to customize and optimize a rigorous deep learning artificial intelligence algorithm for analysis of α-syn pSer129 pathology. Aiforia, Inc. is customizing this AI algorithm with specific input from our team to allow its use in this and our laboratory's other projects. We expect this system to offer significant advantages over densitometry and stereology analyses. Use of the algorithm will allow us to generate extremely accurate estimates of α-syn pathology by sampling entire brain regions (as opposed to extrapolated estimates generated from unbiased sampling of a region generated via stereology software) and will also allow us to quantify total pathology, including Lewy neurites, which will add more information about progression and severity of pathology. Therefore, it will allow us to move past the limitations we have experienced thus far with our densitometry analyses (does not recognize Lewy neurites well; issues with background staining) and with Lewy body stereology (does not account for Lewy neurites at all; regions with sparse pathology require excessive sampling for accurate quantification). Furthermore, the AI platform can analyze entire scanned slides with multiple sections simultaneously in a matter of minutes, astronomically reducing the time load of imaging and analyzing individual sections, which would allow us to study additional brain structures in an unbiased manner. Use of this innovative AI algorithm will be instrumental for our laboratory for accurate quantification and characterization of experimental synucleinopathies, which will significantly improve the rigor of our data. As of this reporting period, we have been finalizing our algorithm customization and have been working with our institute's core to obtain the Zeiss Axio ScanZ.1, which is necessary for use of the AI platform, and which has been in need of servicing by the manufacturer.

– **Y2Q4:** We set out to determine whether our observed lack of differences in α-syn pSer129 presence in olfactory structures could be explained by neuronal death. After staining for neuronal nuclei marker NeuN and counter-staining for Cresyl Violet (Nissl stain), we performed unbiased stereology using Stereo Investigator software. This software program allows estimates of cell populations using unbiased sampling of

a given region. We counted cells positive for both NeuN and Nissl to generate estimates of neuron counts in the AON, which experiences the greatest presence of α-syn pSer129 in our samples. For each mouse AON, six sections, each 40 µm posterior from the previous section, were analyzed using a 1,600 μm^2 counting frame in a 40,000 μm^2 sampling grid.

Specific Aim 2 NeuN stereology analysis of estimated neuron counts in AON:

We found no statistically significant difference in neuron populations between treatment groups (**Figure 4**). Furthermore, there was no correlation between PFF-injected mouse neuron population estimates and α-syn pSer129 presence as analyzed by densitometry. These findings indicate that the lack of difference between treatments in α-syn pSer129 presence in the AON was not due to neuronal death.

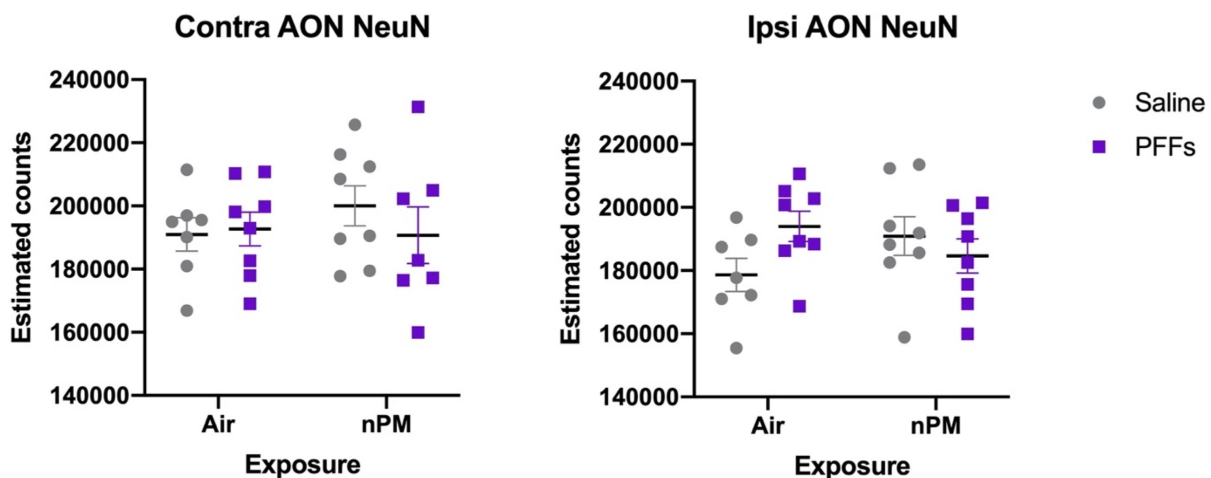


Figure 4: Estimated neuron populations in the ipsi- and contralateral anterior olfactory nucleus (AON) after ten total weeks (three weeks pre-PFF injection, seven weeks post-PFF injection) of nPM or air (control) exposure. Mean ± s.e.m.

Specific Aim 2 pSer129 stereology reanalysis of estimated Lewy body counts in AON:

We also reanalyzed the α-syn pSer129 presence in olfactory areas using the above stereology methods to estimate Lewy body counts in order to validate our densitometry findings. Stereology for Lewy bodies as indicated by pSer129-positive cell body inclusions was performed in the AON. Statistical analysis showed no significant difference in estimates of Lewy body counts ($p > 0.05$) in either ipsi- or contralateral AON (**Figure 5**). However, since unbiased sampling of the AON using stereology methods does not account for the whole brain region, and because pathology in the contralateral AON is more sparse (and pathology in other olfactory regions far more so), the coefficient of error for estimates (Gundersen's; m = 1) in the contralateral AON was high for fully reliable data (CE > 0.1). Rectifying this would require sampling the entire contralateral AON, which can be done more quickly, accurately, and comprehensively with the Aiforia AI algorithm as described above. Notably, there was statistically significant positive correlation between densitometry and stereology data sets, with ipsilateral AON correlation at $r(14) = 0.71$, $p = 0.002$, contralateral AON correlation at $r(14) = 0.80$, $p = 0.0002$, and pooled AON correlation at $r(30) = 0.8816$, $p < 0.00001$ (all samples; Pearson's r). These results further support the densitometry data, validating our original finding (as far as Lewy bodies are concerned) and further suggesting the batch of nPM used in these experiments did not exacerbate α-syn pathology.

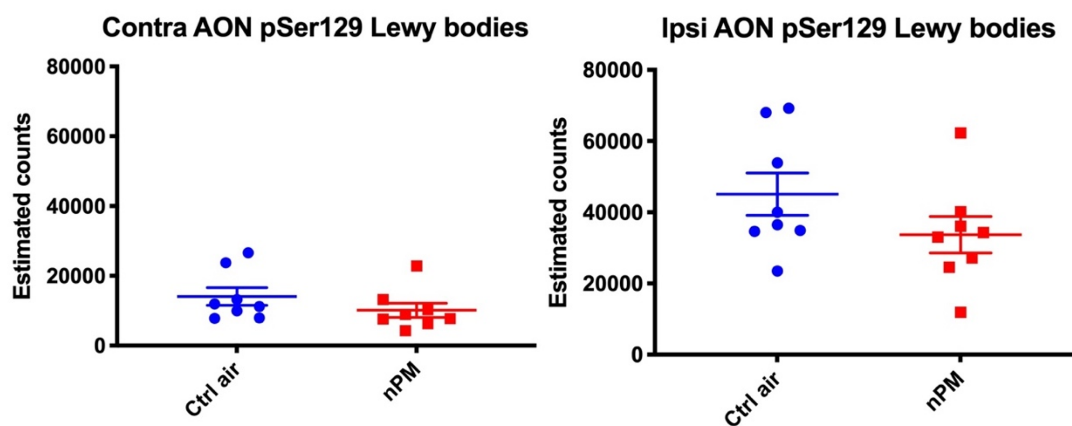


Figure 5: Estimated counts of pathogenic α-syn (pSer129) Lewy bodies in the ipsi- and contralateral anterior olfactory nucleus (AON) after ten total weeks (three weeks pre-PFF injection, seven weeks post-PFF injection) of nPM or air (control) exposure. Mean ± s.e.m.

Ongoing:

Aiforia AI algorithm analysis of α-syn pathology: As of 9/6/19, we have been informed the Axio ScanZ.1 scanner will be serviced in two weeks. As of 9/24/19, The Zeiss Axio ScanZ.1 was replaced and is operational, and we are working with our institute's core to have our slides scanned for upload to Aiforia's cloud.

Specific Aim 2 optimization of immunofluorescent double staining for microglia and inflammatory cytokines: We have been working to optimize immunofluorescent double staining for microglia marker Iba-1 and inflammatory cytokines in this reporting period, but have experienced issues with getting double staining for microglia and cytokines to work (explained in more detail in the Changes/Problems section). The double immunostaining approach affords significant advantages as they will allow microglial colocalization and quantification of expression of the stained inflammatory markers. We are presently optimizing new antibodies for IL-1β, IL-6, and TNF-α using brain tissue from intracerebral LPS-injected positive control animals. These new antibodies have already been published for immunofluorescent staining applications *in vivo*.

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

- Training was provided to postdoctoral fellow Dr. Christopher Tulisiak in unbiased stereology techniques.
- Professional development was provided to all VAI researchers through regular seminars and journal clubs pertaining to neurodegenerative diseases, including Parkinson's disease.

How were the results disseminated to communities of interest?

Posters:

- Tulisiak C. T., Bergkvist L., Cacciottolo M., D'Agostino C., Kuhn E., Meyerdirk L., Steiner J. A., Escobar Galvis M. L., Finch C. E., Morgan T. E., Brundin P. (2019) Effects of airborne pollutants on aggregation of alpha-synuclein and activation of microglia through olfactory structures. Grand Challenges in Parkinson's Disease Abstracts, 24

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

- Complete optimization and analysis of Specific Aim 2 immunofluorescent Iba-1 and inflammatory cytokine double staining
- Scan α-syn pSer129 sections with Axio ScanZ.1 imager and reanalyze using Aiforia AI algorithm
- Continue data analysis and commence manuscript preparation and submission for Specific Aims 1 and 2 experiments
- Commence work on Specific Aim 3 of project

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?***
  - Nothing to Report
- ***What was the impact on other disciplines?***
  - Nothing to Report
- ***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

A. Changes in approach and reasons for change

- As described in the request for modification of Specific Aim 2 submitted 10/10/2018, we altered the Aim 2 exposure to include seven weeks of nPM or air exposure after PFF or PBS injections in addition to the three weeks of pre-injection exposure.

B. Actual or anticipated problems or delays and actions or plans to resolve them

- During this annual reporting period, we recognized the need for more rigorous and accurate methods for identifying and quantifying multiple aspects of α-synucleinopathies, including quantitation of total pathology, adjustment for relative staining intensity, and detection of all types of aggregates, including Lewy neurites, which are not possible to quantify by stereology and densitometry. Furthermore, current state-of-the-art densitometry analysis programs are biased when stained sections have variable staining intensity. We therefore have been working with an external company, Aiforia, Inc., to customize a deep learning AI algorithm for this and all our laboratory's projects. This AI algorithm provides unbiased, comprehensive quantitation of α-syn pathology. While our AI algorithm is now up and running, we have not yet been able to generate scanned images because the necessary equipment has not been operational during the reporting period. We have been informed that our Axio ScanZ.1 equipment was replaced 9/24/19 and that software is now being set up and optimized. We are hoping to have the entire AI algorithm analysis pipeline fully operational by the end of November.
- In this most recent reporting period, we have experienced difficulties in optimization of antibodies for immunofluorescence double staining for both microglia marker Iba-1 and inflammatory markers. Previous attempts at staining for iNOS, IL-6, IL-2β, and TNF-α were unsuccessful or required antigen retrieval steps that are not compatible with microglia-specific Iba-1 antibodies, as the antigen retrieval

steps cause the Iba-1 antibody to recognize neurons as well. We have acquired previously published antibodies for IL-1β, IL-6, and TNF-α that we plan to test during our next reporting period. We are continuing to optimize these antibodies in inflammatory cytokine LPS-injected positive control tissue. If we are still unable to get antibodies for inflammatory markers to work in a double immunostaining context, we will proceed with histology for Iba-1 as reported in Specific Aim 1 experiments above and for inflammatory markers using single epitope staining. Due to limits on available tissue, we have not yet resorted to this course of action.

- Although we continue to analyze the remaining tissues, we have so far observed a modest inflammatory response in all our experiments. We have now indications that not all batches of nPM cause severe inflammatory responses as previously thought, as a report published by our collaborators at USC recently indicates (PMID: 31542466; see also their Y2 annual report, award number W81XWH-17-1-0535). Our collaborators have since generated a new batch of nPM that induces inflammatory responses *in vitro*, which will be used for *in vivo* experiments. Even if our present samples do not indicate inflammation from this batch of nPM, full analyses of these samples will be useful as controls for experiments with inflammatory batches of nPM, greatly increasing our statistical power.

C. Changes that had a significant impact on expenditures

- 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**
  Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

1. Name: Patrik Brundin
   Project role: Principal Investigator/Project Director
   Researcher identifier: ORCID ID: https://orcid.org/0000-0003-2924-5186
   Nearest person month worked: 1.2 cal months (or 10% effort)
   Contribution to Project: Dr. Brundin has performed work to organize and oversee the project, including participating in teleconferences and email correspondence.
   No change

2. Name: Christopher Tulisiak
   Project role: Post-doctoral fellow
   Researcher identifier: ORCID ID: https://orcid.org/0000-0002-0793-1823
   Nearest person month worked: 11 cal months (or 92% effort)

Contribution to Project: Dr. Tulisiak has been driving the processing of the histological samples (staining, imaging, quantitation) and analyses for Specific Aims 1 and 2. He also performed PFF microinjection surgeries for Specific Aim 2 with Ms. Meyerdirk.

3. Name: Jennifer Steiner
   Project role: Senior laboratory manager
   Researcher identifier: ORCID ID: https://orcid.org/0000-0003-0953-1310
   Nearest person month worked: 1.6 cal months (or 13% effort)
   Contribution to Project: Dr. Steiner has performed work to help the Finch laboratory transport materials to and from USC and to coordinate work at VARI necessary to amend and complete the Statement of Work.

4. Name: Emily Kuhn
   Project role: Research technician
   Researcher identifier: ORCID ID: https://orcid.org/0000-0001-9715-7941
   Nearest person month worked: 2.6 (or 22% effort)
   Contribution to Project: Ms. Kuhn performed PFA perfusion and brain collection surgeries for Specific Aim 2 and contributed to sectioning of Specific Aim 2 histological samples.

5. Name: Lindsay Meyerdirk
   Project role: Research technician
   Researcher identifier: ORCID ID: https://orcid.org/0000-0003-4640-9517
   Nearest person month worked: 3.6 (or 30% effort)
   Contribution to Project: Ms. Meyerdirk performed PFF microinjection surgeries for Specific Aim 2 with Dr. Tulisiak. She also contributed to sectioning of Specific Aim 2 histological samples.

6. Name: Liza Bergkvist
   Project role: Post-doctoral fellow
   Researcher identifier: ORCID ID: https://orcid.org/0000-0001-7433-2647
   Nearest person month worked: 2.4 (or 20% effort)
   Contribution to Project: Dr. Bergkivst processed histological samples (sectioning, staining, imaging, quantitation) for Specific Aim 1 pSer129 analysis and is aiding in Specific Aim 2 inflammatory cytokine antibody optimization and general analyses.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes; see below:

Recently Completed Support

N/A (Brundin, P.) 9/1/2017 - 5/31/2018 0.24 Cal Mths or 2% Effort
Cure Parkinson's Trust $97,242
Defining Levels of TDZs Active Metabolites in CSF and Brain Mitochondria – Is MSDC-0160 a more pertinent drug than pioglitazone?
The major goal of this project is to determine if MSDC-0160 a more pertinent drug than pioglitazone. Specific aims: 1) Measure MSDC-0160 and pioglitazone and their metabolites in plasma and CSF as well as in the brain-mitochondrial fraction to determine levels of exposure in brain. 2) Determine the minimal concentration of MSDC-0160 and pioglitazone, and active metabolites, required for rescuing mitochondrial

activity in cultured neurons under neurotoxic conditions.
Agency contact: Helen Matthews; e-mail: helen@cureparkinsons.org.uk.
Role: PD/PI
No overlap

R43NS097105 (Gregor) 8/1/2016 – **1/31/2019** 0.24 Cal Mths or 2% Effort
NIH/NINDS via GISMO Therapeutics $110,054
Glycosaminoglycan-Interacting Small Molecule (GISMO) As Parkinson's Therapeutics
The objective of this phase 1 study is to develop new disease modifying drugs for Parkinson's disease (PD), targeting the interaction between alpha-synuclein and its cell surface receptor, heparan sulfate glycosaminoglycans
Specific aims: 1) Evaluate GISMO Lead Compounds in neuronal cell culture assays for potency and safety. 2) Optimization of Selected Lead Series. 3) Evaluation of pharmacokinetics of lead compounds. 4) Evaluation of Three Selected Lead Compounds in mouse model of PD associated with α-syn transfer and aggregation.
Agency Contact: Elizabeth Conklin; email: conklinee@ninds.nih.gov
Role: PD/PI
No overlap

12253 (Brundin, P and Pfeifer, G) 11/2/2016 – **03/29/2019** 0.24 Cal Mths or 2% Effort
MJFF $200,000
Upregulation of Autophagy by Tet3-mediated 5-methylcytosine – Relevance to PD
The major goal of this project is to determine the role of Tet3 in the regulation of autophagy and its relevance to Parkinson's disease.
Specific aims: 1) To demonstrate diminished functionality of the TET3FL oxidation pathway at lysosomal genes during normal aging. 2) To demonstrate decreased functionality of the TET3FL oxidation pathway at lysosomal genes in PD. 3) To manipulate TET3FL in a cultured cell system and monitor changes in LAS and α-syn aggregation. 4) To manipulate Tet3FL in vivo and monitor changes in LAS and α-syn aggregation.
Agency contact: Julia Keefe; e-mail: jkeefe@michaeljfox.org.
Role: Co-PD/PI
No overlap

MSDC-0160-GLP-1 (Brundin, P.) 8/1/2017 - **7/31/2019** 0.6 Cal Mths or 5% Effort
Cure Parkinson's Trust $216,719
Defining The Effects Of Combining A GLP-1 Analogue And An Insulin Sensitizer In Models Of PD-Like Neuropathology (No Cost Extension)
The major goal of this project is to define the effects of combining a GLP-1 analogue and an insulin sensitizer in models of PD- like neuropathology.
Specific aim: To determine if the effect of combining a GLP-1 analogue (exendin-4) and an insulin sensitizer (MSDC-0160) will be synergistic resulting in greater neuroprotection when compared to single treatments.
Agency contact: Helen Matthews; e-mail: helen@cureparkinsons.org.uk.
Role: PD/PI
No overlap

N/A (Brundin, P.) 3/22/2017 - **9/21/2019** 0.00 Cal Mths or 0% Effort
Lundbeck $420,155
SPRA: Optimizing the olfactory bulb injection model to be optimal suitable for testing efficacy of antibodies

The major goal of this project is to use a mouse model of prodromal PD to define the efficacy of antibodies directed against pathological alpha-synuclein.
Specific aims: 1) To assess the seeding activity of human alpha-synuclein fibrils produced in VARI in primary neurons and to confirm that H Lundbeck A/S (HLu) antibodies recognize these fibrils. 2) To assess the seeding activity of human alpha-synuclein fibrils in vivo in both wt and transgenic F28 mice. 3) To assess mechanism of antibody activity on spreading of alpha-synuclein pathology. 4) To assess antibody effect on primary alpha-synuclein pathology development and olfactory dysfunction. 5) To assess antibody effect on spreading of secondary alpha-synuclein pathology and dysfunction associated with secondary pathology.
Agency contact: Karina Fog; email: kape@lundbeck.com
Role: PD/PI
No overlap

Changes to Current Research Support
(THIS AWARD)
W81XWH-17-1-0534 (Brundin, P) 9/1/2017 - 8/31/2021 1.20 Cal Mths or 10% Effort
DoD $1,417,473
Airborne pollutants as triggers of Parkinson's disease via the olfactory system
The major goal of this project is to determine the influence of air pollutants in the development of PD.
Specific aims: 1) Determine the effects of nPM exposure after microinjection of fibrillar α-syn in the OB. 2) Determine the effects of nPM exposure prior to the microinjection of fibrillar α-syn in the OB. 3) Define the effects of systemic administration of ibuprofen on the development of α-syn pathology. 4) Define the effects of systemic administration of MSDC-0160 on the development of α-syn pathology.
Agency contact: Christopher Meinberg; email: christopher.l.meinberg.civ@mail.mil
Role: PD/PI
No overlap

N/A (Brundin, P.) 1/11/2016 - 1/10/2020 0.00 Cal Mths or 0% Effort
Hoffmann-La Roche $107,010
SPRA: The role of inflammation in alpha-synuclein propagation w/visiting scientist Nazia Maroof (***No Cost Extension***)
The major goal of this project is to determine whether aSyn pathology can propagate from the ENS to the Central Nervous System (CNS) and what the role of the immune system may be in this process.
Specific aims: 1) To determine whether inflammation following acute (1 week)/chronic (4 weeks) DSS colitis has a long term impact on aSyn accumulation in the ENS and CNS and what role it plays in aSyn propagation from ENS to CNS. 2) To ascertain whether aSyn isolated from faecal samples from PD patients and transgenic aSyn overexpressing mice which have been subjected to DSS colitis, causes seeding and propagation of aSyn aggregate pathology through the nervous system following injection into the olfactory bulb. 3) To investigate whether colitis in early life of Thy1-(A30P)aSyn tg mice leads to aSyn pathology and gliosis in brain over longer period of time. 4) To explore epigenetic changes in nuclei of enteric nerves after DSS colitis in Thy1-(A30P)aSyn tg mice.
Agency contact: Markus Britschgi; email: markus.britschgi@roche.com
Role: PD/PI
No overlap

3R01DC014443-04S2 (Wesson) 7/1/2018 - 6/30/2020 0.48 Cal Mths or 4% Effort
NIH/NIDCD via U of Florida $142,500 (No Cost Extension)
Inter-Regional Coding of Odor Valence by Neural Ensembles

The major goal is to determine neuropathological changes associated with olfactory dysfunction in a mouse model of Alzheimers.
Specific Aim: to define/quantify the levels of brain neuropathology in a mouse model of Alzheimer's disease.
Agency Contact: Hoai Doan; email: hoaid@nidcd.nih.gov
Role: Subaward PD/PI
No overlap
*New since last report

22044 (Brundin, P) 10/1/2018 - 9/30/2019 0.00 Cal Mths or 0% Effort
East Tennessee Fdn $25,000
Defining the influence of a genetic risk factor in the development of Parkinson's Disease
The major goal of this project is to determine the negative effects of external insults in these mice and to initiate experiments aiming to counteract the negative effects of lacking ACMSD using a novel drug.
Specific aims: 1) Define the effects of the lack of ACMSD upon exposure to insults known to induce nigral neurodegeneration. 2) Determine the tolerability and brain penetrance of a novel drug to counteract the negative effects of deleting the ACMSD gene.
Agency contact: Jan Elston; e-mail: jelston@etf.org
Role: PD/PI
No overlap
*New since last report

R21NS106078-01A1 (Brundin & Kordower) 1/15/2019 - 12/31/2020 1.2 Cal Mths or 10% Effort
NIH/NINDS $920,918
Combining synucleinopathy and mitochondrial deficits in a novel mouse model of Parkinson's disease
The major goal of this project is to develop a new mouse model of PD combining mitochondrial deficits and alpha synuclein pathology.
Specific aims: 1) Determine effects of combining mitochondrial dysfunction and α-syn aggregation on neurodegeneration. 2) Determine effects of combining mitochondrial dysfunction and α-syn aggregation on motor behaviors relevant to PD.
Agency contact: Crystal L Anderson; email: andersoncl@mail.nih.gov
Role: PD/PI
No overlap
*New since last report

N/A (Brundin, P & Brundin, L) 2/1/2019 – 1/31/2022 0.00 Cal Mths or 0% Effort
Peter C & Emajean Cook Foundation $225,000
Defining the role of a genetic risk factor in the development of Parkinson's disease.
The major goal of this project is to define the effects of up– and down–regulating ACMSD in vivo.
Specific aims: Not applicable
Agency contact: Carrie Boer, 2900 Charlevoix Drive SE Ste 130 Grand Rapids MI 49546
Role: PD/PI
No overlap
*New since last report

NA (Brundin, P) 3/1/2019 - 2/28/2021 0.00 Cal Mths or 0% Effort
Farmer Family Foundation $190,172
Infections as triggers of Parkinson's disease (iPark)
The major goal of this project is to identify bacterial and viral pathogens that can act as triggers of Parkinson's disease (PD) and to characterize their mechanism of action.
Specific aims: Not applicable

Agency contact: Available upon request
Role: SubProject PI
No overlap
*New since last report

What other organizations were involved as partners?

None identified outside of our funded DoD collaborations.

8. SPECIAL REPORTING REQUIREMENTS

See attached Quad Chart.

9. APPENDICES

N/A

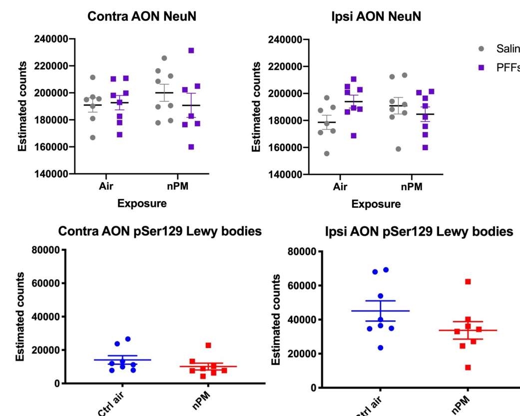
Project Title: Airborne Pollutants as Triggers of Parkinson's Disease via the Olfactory System
Log Number: PD 160021 Annual Report Year 2
Award Number: W81XWH-17-1-0534

PI: Brundin, Patrik **Org:** Van Andel Research Institute **Award Amount:** $746,039/Direct

Study Aims

1. Determine the effects of nPM exposure *after* microinjection of fibrillar α-syn in the OB.
2. Determine the effects of nPM exposure *before* microinjection of fibrillar α-syn in the OB.
3. Define the effects of systemic administration of ibuprofen on the development of α-syn pathology.
4. Define the effects of systemic administration of MSDC-0160 on the development of α-syn pathology.
5. Examine the effect of long-term exposures to ambient $PM_{2.5}$ and NO_2 on hyposmia.
6. Examine whether early PD pathogenesis is exacerbated by ambient air pollutants.
7. Examine whether lifetime use of NSAIDs, ibuprofen in particular, modify potential adverse effects of air pollutants on hyposmia.

Approach: This project ranges from *in vivo* studies, to elucidate the influence of exposure to airborne pollutants on the development of α-syn pathology, to epidemiological studies, to unravel the contribution of relevant factors in PD-like long-time exposure to airborne pollutants, genetic risk score or use of NSAIDs (as well as the interactions among these factors).



Accomplishments: Stereology for estimated neuron (top) and Lewy body (bottom) counts. No statistically significant difference was observed in either analysis ($p > 0.05$). These findings may indicate the batch of nPM used was ineffective at exacerbating alpha-synucleinopathy or inducing inflammation (analysis ongoing). More potent pro-inflammatory nPM is being generated, and the current data, where the nPM unexpectedly did not induce inflammation, can serve as controls to increase statistical power.

Timeline and Cost

Activities CY	17/18	19	20	21
Study Prep/Specific Aim 1	$177,530			
Specific Aim 2 (see goals/milestones)		$183,808		
Specific Aim 3 (see goals/milestones)			$192,162	
Specific Aim 4 (see goals/milestones)				$192,539
Estimated Budget ($746,039)	**$177,530**	**$183,808**	**$192,162**	**$192,539**

Updated: 9/24/19

Goals/Milestones

CY17: 1) ☑ Obtain IACUC approval at USC; 2) ☑ Generation of PFFs
CY18: 1) ☑ Generation of PFFs; 2) ☑ ACURO regulatory approval; PFFs, nPM ready to be used; 3) ☑ Inject mice with PFFs at USC and nPM exposure (aim 1); 4) ☑ Histological analyses (aim 1): pSer129 and Iba-1 quantification
CY19: 1) ☑ nPM exposure and inject mice with PFFs (aim 2); 2) Histological analysis (aim 2): ☑ pSer129 and NeuN; Iba-1 and inflammatory cytokine (ongoing); 3) data analysis/manuscript prep and submission
CY20: 1) Inject mice with PFFs (aim 3); 2) Histological analysis (aim 3); 3) data analysis/manuscript prep and submission; 4) Inject mice with PFFs (aim 4); 5) Histological analysis (aim 4)
CY21: 1) Histological analysis (aim 4); 2) data analysis/manuscript prep and submission

Comments/Challenges/Issues/Concerns

- NA

Budget Expenditure to Date

Projected Expenditure: $361,338
Actual Expenditure: $241,033