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TITLE: Airborne Pollutants as Triggers of Parkinson's Disease via the Olfactory System

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CONTRACTING ORGANIZATION: Van Andel Research Institute
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14. ABSTRACT
We are interested in determining whether ambient air pollutants impact the development of Parkinson's disease (PD) by increasing α-synuclein pathology via inflammation. In this aim we injected α-synuclein (α-syn) pre-formed fibrils (PFF) in the right olfactory bulbs of mice to model PD then exposed them to nano-particulate matter (nPM) for four weeks after injections. Ten weeks later, we euthanized the mice. We first confirmed that PFF injections induced the expected phosphorylated α-syn pathology throughout olfactory areas, and we are presently determining whether nPM exposure worsened that pathology via neuroinflammation. In summary, we observe increased phosphorylated α-syn pathology in the cortical amygdala after nPM exposure.

15. SUBJECT TERMS
Pre-formed fibrils (PFFs), α-synuclein (α-syn), 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.

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):

Specific Aim 1: Determine the effects of exposing mice to nPM after triggering of PFF pathology (Months 6-16)

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

    Validation experiment accomplished (n=32), 10/12/17 (Q1)

    Aim 1 injections (n=64) accomplished 1/18/18 (Q2)

2. Expose C57BL/6J mice to nPM.

    Validation experiment accomplished (n=32), 11/09/17 (Q1)

    Aim 1 exposure (n=64) accomplished 2/14/18 (Q2)

Milestones in this reporting period:

1. Receipt of validation experiment processed brains at VARI, **accomplished (n=32), 11/21/17 (Q1).**
2. Receipt of Aim 1 full experiment processed brains at VARI, **completed on 5/1/18 (Q3).**
3. Histological analyses (starts after 1 month for sectioning), for validation experiment **completed at VARI (Q3).**
4. Histological analyses of pSer129 for Aim 1 full experiment, **completed at VARI (Q4).**
5. Histological staining of Iba-1 for Aim 1 full experiment (ongoing, Months 10-16) **(Q4)**.

6. Biochemical analyses for full experiment, **completed at USC (Q4).**

Specific Aims 2, 3 & 4: **not yet initiated**

What was accomplished under these goals?

1) Major activities:

1) Preparation of PFFs at VARI (Q1).
2) Prepare for first collaborative study involving personnel from VARI and USC (Q1).
3) Inject validation experiment C57BL/6J mice with PFFs at USC (by VARI personnel) (Q1).
4) Expose validation experiment C57BL/6J mice to nPM at USC (by USC personnel) (Q1).
5) Collect validation experiment brains at USC and mail to VARI (Q1).
6) Inject full experiment C57BL/6J mice with PFFs at USC (by VARI personnel) (Q2).
7) Expose full experiment C57BL/6J mice to nPM at USC (by USC personnel) (Q2).
8) Histological analysis of validation experiment brains (by VARI personnel) (Q2).
9) Collect full experiment brains at USC and mail to VARI (Q3)
10) Histological analysis of full experiment brains (by VARI personnel) (Q3, Q4).

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:

Q1: In September 2017, before initiating our first collaborative study VARI's PI and researchers teleconferenced with USC PIs and researchers to discuss and refine our strategy to complete the Statement of Work. At the end of the teleconference, we decided to exclude one of the experimental groups: monomeric alpha-synuclein. This group was not necessary, according to our rationale, as monomeric alpha-synuclein will not aggregate and spread into the brain (which is our primary outcome). Therefore, this group was determined to be redundant with the saline control group and was removed.

Additionally, as VARI and USC researchers had never worked together previously and since the study required Dr. Nolwen Rey (VARI) to travel to USC to perform the precise surgical injections, we wanted to first perform injections into a smaller set of animals. This validation study was needed to ensure that all procedures could be properly executed at USC, by Dr. Nolwen Rey and USC researchers. Therefore, our initial study was on 32 mice, which were 16 fibrillar alpha-synuclein injected + 16 saline injected.

Q2: In December 2017, Dr. Nolwen Rey (VARI personnel) reported to VARI and USC personnel preliminary results of the 32-mouse validation experiment: phospho-alpha-synuclein-specific pathology was observed in 6 PFF-injected mice, and no pathology was observed in 6 PBS- injected mice. Subsequently,

remaining tissue from the validation experiment was processed to detect phosphorylated serine 129 (pSer129) of pathological α-synuclein (α-syn) pre-formed fibrils (PFFs). Further analysis was completed in Q3.

As stated in the Q1 report, we planned to initiate another collaborative study with USC to inject into mice and obtain tissues for the analyses of all of the proposed endpoints in Aim 1. In January 2018, Dr. Nolwen Rey and Ms. Lindsay Meyerdirk (VARI personnel) traveled to USC to perform PFF injections into 70 C57Bl/6J mice, with USC personnel. The mice were subsequently exposed to nPM by USC personnel for 4 weeks. At ten weeks after nPM exposure, the mice were scheduled to be euthanized and processed for analysis at USC and VARI, according to the Statement of Work.

Q3: Remaining tissue from the validation experiment (Q1-Q2) was processed to detect fibrillar α-syn pSer129 pathology. Further analysis was completed during this reporting period, and α-syn pSer129 pathology was detected, as expected, in all of the remaining PFF-injected mice.

As stated in the Q2 report, VARI personnel performed PFF injections into 70 C57Bl/6J mice, at USC, with USC personnel, in January 2018. The mice were subsequently exposed to nPM by USC personnel for 4 weeks. Ten weeks after nPM exposure, the mice were euthanized and processed for analysis at USC and VARI, according to the Statement of Work. VARI staff received 32 brains on May 1, 2018, and has sectioned all brains, stained a full series of brain sections for fibrillar α-syn pSer129 pathology, and imaged these sections. We preliminarily concluded that α-syn pSer129 pathology was detected, as expected, in relevant brain areas in all of the PFF-injected mice. We are still quantifying the α-syn pathology in order to determine whether the nPM exposure exacerbated the pathology development.

Q4: Staining for α-syn pSer129 and for microglia marker Iba-1 was completed per Dr. Brundin's laboratory's standard immunohistochemistry protocols. α-syn pSer129 analyses were conducted using an ImageJ plugin for quantification of the pixel area surpassing a threshold intensity (α-syn pSer129-stained tissue), while Iba-1 cell counts are being conducted manually using an ImageJ cell counter (in progress).

pSer129 staining:
By comparing air-exposed controls to the nPM-exposed experimental group in experimental PFF- and control PBS-injected animals, we were able to quantify fibrillar α-syn pSer129 staining as a function of stained area across the whole of the image's tissue area and to compare spread of pathogenic fibrillar α-syn from the injection site to other areas of the brain. We have previously demonstrated that animals injected with PFFs in the olfactory bulb (OB) demonstrate fibrillar α-syn pathology in the OB, anterior olfactory nucleus (AON), and cortical amygdala (CoAM). PBS-injected control animals do not demonstrate any significant α-syn pSer129 pathology, and all PBS-injected groups demonstrate lower α-syn pSer129 staining compared to their PFF-injected counterparts, validating that PFF injections are necessary for development of fibrillar α-syn pathology in our model. A low level of background signal appears to the same extent in all brain regions analyzed for PBS-injected animals, and there is no significant difference in α-syn pSer129 signal between PBS-injected control animals exposed to nPM or air (Figure 1).

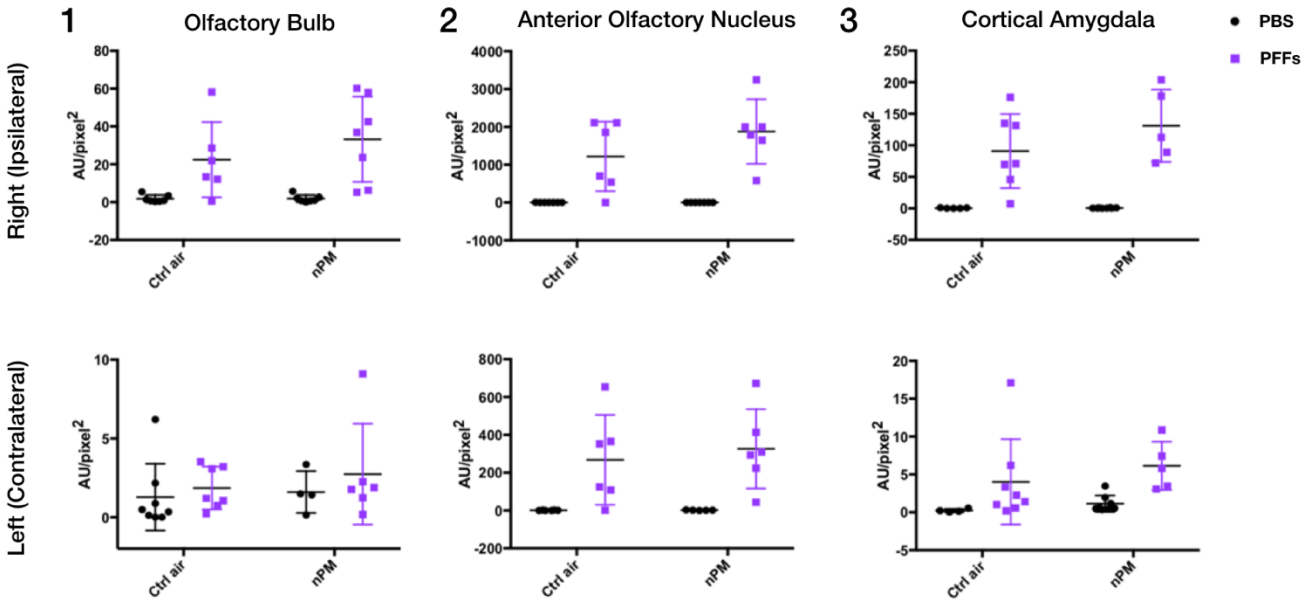


Figure 1: Validation of fibrillar α-syn pathology (phosphorylated serine 129; pSer129) in olfactory areas after four weeks of nPM or air (control) exposure in right (ipsilateral to injection) and left (contralateral to injection) sides of PBS- and PFF-injected mouse brains. For all olfactory areas, significantly higher α-syn pSer129 pathology was observed in the right (ipsilateral) side of PFF-injected brains compared to PBS-injected brains of both air- and nPM-exposed mice ($p < 0.05$), whereas the left (contralateral) side shows low levels of α-syn pSer129 pathology (ns). 1) Olfactory bulb. 2) Anterior olfactory nucleus. 3) Cortical amygdala. AU: arbitrary units; ns: not significant; PBS: phosphate buffered saline; PFFs: pre-formed fibrils; Ctrl air: air-exposed control group; nPM: nano-particulate matter group.

Comparisons of the effects of nPM on development and spread of synucleinopathy in specific brain regions can be found in Figure 2. In summary, the CoAM demonstrates a significant increase in α-syn pSer129 staining on the PFF-injection side compared to the contralateral side in both the nPM- and air-exposed groups. Most interestingly, pooling α-syn pSer129 staining data from both ipsi- and contralateral sides of the CoAM reveals a small but significant increase in fibrillar α-syn pathology in the nPM-exposed group compared to the air-exposed group (Figure 2.3). Furthermore, pooling α-syn pSer129 staining data from ipsi- and contralateral sides for all three brain regions (OBs, AON, CoAM) also reveals a small but significant increase in fibrillar α-syn pathology in the nPM-exposed group compared to controls, though this increase may be driven by the CoAM dataset (Figure 2.4). Nevertheless, these data suggest a significant effect of nPM exposure to spread of pathological fibrillar α-syn, particularly in deep brain structures like the CoAM, even after ten weeks since final nPM exposure. Analysis of the OBs (Figure 2.1) and AON (Figure 2.2) alone, however, does not demonstrate a significant nPM-exposure related increase in fibrillar α-syn pSer129 pathology, though there is a trend toward elevated α-syn pSer129 pathology in the ipsilateral side compared to the contralateral in both air- and nPM-exposed groups.

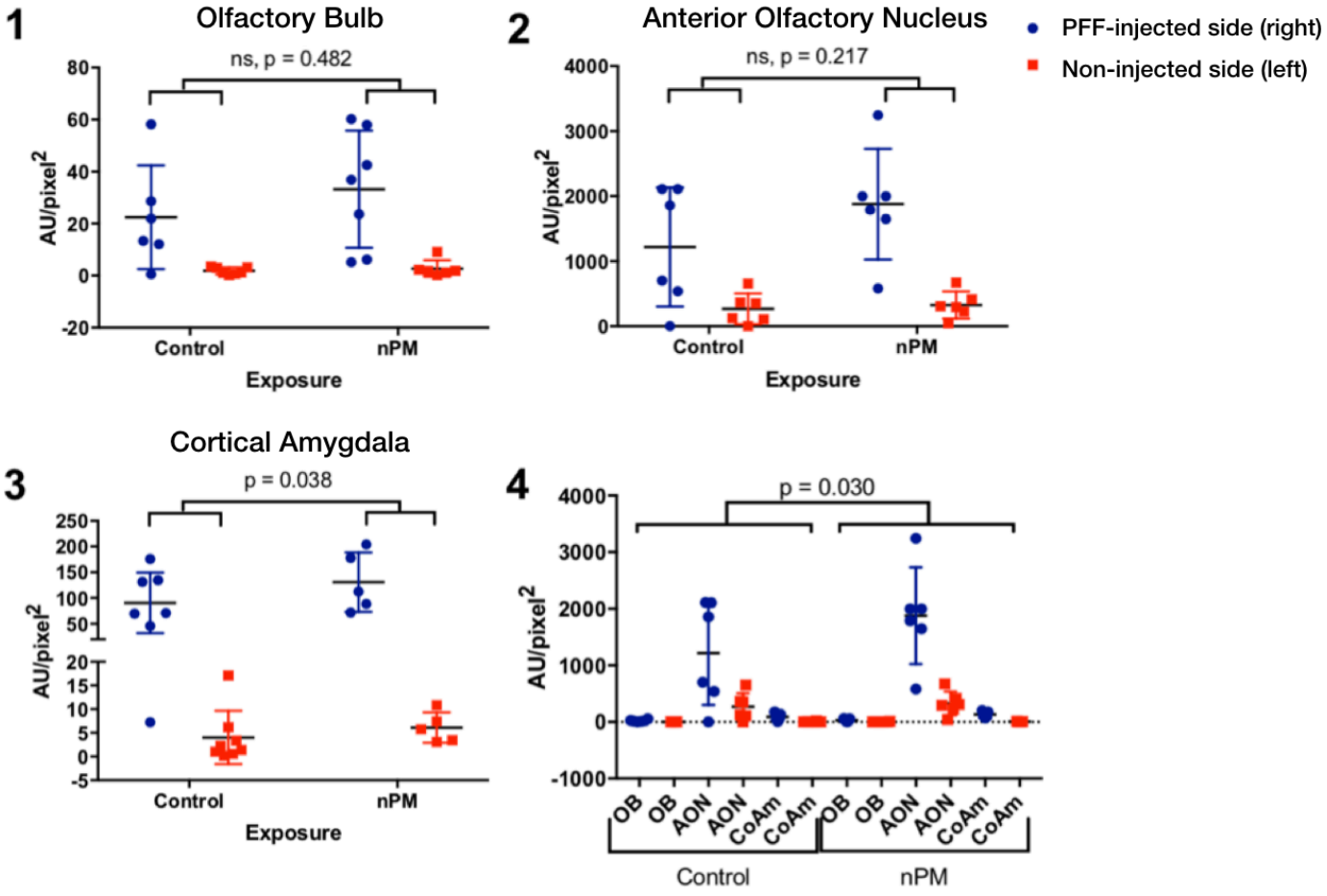


Figure 2: Fibrillar α-syn pathology (phosphorylated serine 129) in olfactory areas after four weeks of nPM or air (control) exposure. 1) Olfactory bulbs; no significant difference between nPM and control groups. 2) Anterior olfactory nucleus; no significant difference between nPM and control groups. 3) Cortical amygdala; pooling of data from PFF-injected side and contralateral side yields significant difference between nPM and control groups ($p = 0.038$). Significant difference between PFF-injected and contralateral sides also observed in both nPM and control groups ($p < 0.05$). 4) Pooling of data from PFF-injected and contralateral sides of the brain from all brain regions demonstrates a significant increase in fibrillar α-syn pathology in nPM exposed olfactory areas compared to controls ($p = 0.030$). AU: arbitrary units; ns: not significant; PFF: pre-formed fibrils; Control: air-exposed control group; nPM: nano-particulate matter group; OB: olfactory bulb; AON: anterior olfactory nucleus; CoAm: cortical amygdala.

Iba-1 staining:
Staining has been completed. Imaging and analysis for Iba-1 will be finished before the next quarterly report.

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

- Training was provided to postdoctoral fellow Dr. Christopher Tulisiak in stereotaxic surgery and microinjection 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?

- Nothing to Report

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

- Complete analysis of Aim 1 Iba-1 staining and morphological characterization of microglia
- Commence work on Aim 2 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

- **Changes in approach and reasons for change**
  - Nothing to Report
- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - Nothing to Report
- **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.

2. Name: Christopher Tulisiak
Project role: Post-doctoral fellow
Researcher identifier: ORCID ID: https://orcid.org/0000-0002-0793-1823
Nearest person month worked: 1.44 cal months (or 12% effort)
Contribution to Project: Dr. Tulisiak started work on the project on July 1, 2018 and has been driving the processing of the histological samples (staining, imaging, quantitation) of Aim 1.

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

R21NS093993 (Brundin) 2/15/2016 - 1/31/2018 0.60 Cal Mths or 5% Effort
NIH/NINDS $125,000
Does Microglial Activation Influence Propagation of Alpha-synuclein Pathology
The long-term goal of this project is to establish how inflammation can interfere with the transfer of a-synuclein in vivo.
Specific aims: 1) Define whether absence of microglia impacts neuron-to-neuron transfer of α-syn. 2) Determine how microglia activated by either IL-4 or LPS affects the rate of neuron-to-neuron transfer of α-syn.
Agency contact: Ashley Dash; e-mail: dasher@mail.nih.gov.
Role: PD/PI
No overlap

D-PUFAS (N/A) (Brundin and Van der Ploeg) 10/15/2015 - 6/30/2017 0.60 Cal Mths or 5% Effort
Cure Parkinson's Trust $147,514
Preclinical Evaluation of D-PUFAs as a Therapeutic Intervention for PD
The major goal of this project aims to test the hypothesis that RT001 will protect neurons against degeneration in PD models by inhibiting lipid peroxidation.
Specific aims: 1) Define the effect of D-PUFAs (administered as a dietary supplement) in a mouse model exhibiting specific nerve cell death akin to that seen in PD. 2) Optimize the administration of D-PUFAs and determine the levels of drug exposure and protection in relevant brain regions, and develop biomarkers of drug (D-PUFA) exposure, essential for choosing dosages in clinical trials.
Agency contact: Helen Matthews; e-mail: helen@cureparkinsons.org.uk.
Role: PD/PI
No overlap

11451 (Brundin, P and Brundin, L) 12/11/2015-12/10/2016 0.24 Cal Mths or 2% Effort
Michael J Fox Foundation $100,000
Validating the neuroprotective enzyme ACMSD as a novel therapeutic target in Parkinson's disease by viral vector mediated overexpression.
The major goal of this project is to validate ACMSD in models of PD.

Specific aim: To define the effect of selective viral vector-mediated overexpression of ACMSD (in neurons or microglia) in a localized neuroinflammation model, induced by intranigral LPS injection, as a key step in the validation of ACMSD as a therapeutic target for PD.
Agency contact: Adria Martig; e-mail: amartig@michaeljfox.org.
Role: PI
No overlap

Current Research Support

R43NS097105 (Gregor) 8/1/2016 - 1/31/2019 0.12 Cal Mths or 1% Effort
NIH/NINDS via GISMO Therapeutics $0
Glycosaminoglycan-Interacting Small Molecule (GISMO) As Parkinson's Therapeutics (No Cost Extension)
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 (a-syn) and its cell surface receptor, heparan sulfate glycosaminoglycans (HS-GAG).
Agency contact: Elizabeth Conklin; email: conklinee@ninds.nih.gov
Role: Subaward PD/PI
No overlap

R01DC016519 (Brundin, P.) 7/1/2017 - 6/30/2022 1.80 Cal Mths or 15% Effort
NIH/NIDCD $381,906
Linking Synucleinopathy and Dysfunction of Olfactory Pathways
To establish how the progressive spreading of aggregated a-synuclein from the olfactory bulb to other olfactory structures causes loss of olfaction.
Specific aims: 1) Determine the cellular mechanisms associated with synucleinopathy that underlie olfactory deficits. 2) Establish the effects of immunotherapy on olfactory deficits associated with the progression of synucleinopathy. 3) Define the role of microglia in the development of synucleinopathy and olfactory deficits.
Agency contact: Maria Garcia; email: mg421s@nih.gov
Role: PD/PI
No overlap

12253 (Brundin, P) 11/2/2016 - 11/1/2018 0.24 Cal Mths or 2% Effort
MJFF $80,000
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) Demonstrate diminished functionality of the TET3FL oxidation pathway at lysosomal genes during normal aging. 2) Demonstrate decreased functionality of the TET3FL oxidation pathway at lysosomal genes in PD. 3) Manipulate TET3FL in a cultured cell system and monitor changes in LAS and α-syn aggregation.
Agency contact: Allesia Krank; email: akrank@michaeljfox.org.
Role: PD/PI
No overlap

MSDC-0160-GLP-1 (Brundin, P.) 8/1/2017 - 1/31/2019 0.24 Cal Mths or 2% Effort
Cure Parkinson's Trust $108,359
Defining the Effects of Combining a GLP-1 Analogue and an Insulin Sensitizer in Models of PD-Like Neuropathology
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: We hypothesize that by combining a GLP-1 analogue (exendin-4) and an insulin sensitizer (MSDC-0160), their effects 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

R21NS105436 (Brundin, P.) 1/1/2018 - 12/31/2019 0.36 Cal Mths or 3% Effort
NIH/NINDS $132,250
Promoting Survival of Dopamine Neurons in Models of Parkinson's Disease Using a Novel Transcriptional Regulator
The major goal of this project is to define how up regulating a novel transcriptional cascade (PM-Nato3) influences the survival of dopamine neurons in models of Parkinson's disease.
Specific aims: 1) Define the effects of PM-Nato3 expression in MPP+ and α-synuclein toxicity models of PD using cultured human DA neurons. 2) Define the effects of PM-Nato3 expression in En1 haploinsufficient and α-synuclein animal models of PD.
Agency contact: Vicky Haines; email: vhaines@mail.nih.gov
Role: PD/PI
No overlap

What other organizations were involved as partners?

None identified outside of our funded DoD collaborations.

8. SPECIAL REPORTING REQUIREMENTS

See attached Quad Chart.

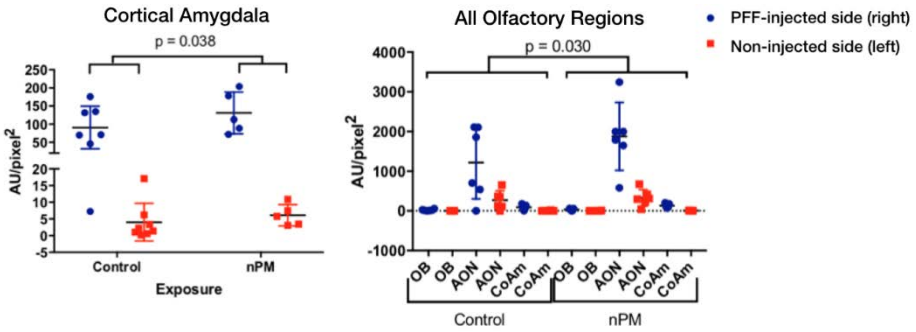
Project Title: Airborne Pollutants as Triggers of Parkinson's Disease via the Olfactory System
Log Number: PD 160021 Annual Report Year 1
Award Number: W81XWH-17-1-0534

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 prior to 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: Fibrillar α-syn pathology (phosphorylated serine 129) in olfactory areas after four weeks of nPM or air (control) exposure. *Left:* Cortical amygdala; pooling of data from PFF-injected side and contralateral side yields significant difference between nPM and control groups ($p = 0.038$). Significant difference between PFF-injected and contralateral sides also observed in both nPM and control groups ($p < 0.05$). *Right:* Pooling of data from PFF-injected and contralateral sides of the brain from all brain regions demonstrates a significant increase in fibrillar α-syn pathology in nPM exposed olfactory areas compared to controls ($p = 0.030$). AU: arbitrary units; PFF: pre-formed fibrils; Control: air-exposed control group; nPM: nano-particulate matter group; OB: olfactory bulb; AON: anterior olfactory nucleus; CoAm: cortical amygdala.

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: 9/28/18

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 (aim 1); 4) Histological analyses (aim 1): ☑ pSer129 quantification; Iba-1 quantification is ongoing
CY19: 1) Inject mice with PFFs (aim 2); 2) Histological analysis (aim 2); 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: $177,530
Actual Expenditure: $66,124