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TITLE: Novel Exposomics and Extracellular Vesicle Biomarkers to Unravel Gene-Environment Interactions and Mechanisms of Neurodegeneration in Parkinson's Disease

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CONTRACTING ORGANIZATION: Columbia University Medical Center, New York, NY

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14. ABSTRACT

Our goal is to characterize the synergistic contribution of the most common but incompletely penetrant genetic cause of Parkinson's disease (PD), LRRK2, and exposure to organic toxicants and neurotoxic metals suspected to be implicated in PD. Our Specific Aims (SA) are to SA-1: Use blood-borne extracellular vesicles (EVs) from the brain of PD patients to elucidate their exposome and metallome; and determine if individual toxicants or mixtures, independently or synergistically with LRRK2 activation status, represent predictive biomarkers of disease. So far, we immunoisolated EVs from glial (GLAST) origin from plasma of healthy controls (n=65); sporadic PD (n=65); unaffected carriers LRRK2+/PD- (n=8); affected LRRK2+/PD+ (n=35) and are currently validating the isolation of neuronal-derived EVs (ATP1A3) before running our LRRK2 status, metallomics and exposomics analyses. SA-2: Identify GxE interactions relevant to PD-linked DA neurodegeneration and LRRK2 pathology. This first year, we amplified 6 induced pluripotent stem cell lines from LRRK2 PD patients and age/sex-matched controls. We differentiated these lines in either dopaminergic or astrocyte cultures and are currently screening different metal and organic toxicity on these cells. Our preliminary data suggest that control and PD LRRK2 astrocytes may have different capacity of eliminating excess of certain metals (Mn, As and Cu) via the EV pathway with potential neurotoxic consequences.

15. SUBJECT TERMS

Parkinson's disease; LRRK2; extracellular vesicles; metals; organic toxicants; exposomics, metallomics; biomarkers; gene-environment interaction; neurotoxicity

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

The progressive loss of dopamine (DA)-producing neurons in the substantia nigra pars compacta underlies the debilitating motor symptoms characteristic of Parkinson's disease (PD). While the exact pathogenesis remains unclear with 90% of PD cases being sporadic, both environmental as well as genetic factors are thought to contribute. The focus of this proposal is to characterize the potential synergistic contribution of the most common but incompletely penetrant genetic cause of PD; leucine-rich repeat kinase 2 (LRRK2), and exposure to organic toxicants and neurotoxic metals suspected to be implicated in PD etiology. Our goals are also to provide novel reliable non-invasive brain biomarkers of environmentally linked PD and its progression, as well as new *in vitro* and preclinical models to advance neuroprotective strategy development.

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

Parkinson's disease; LRRK2; extracellular vesicles; metals; organic toxicants; exposomics, metallomics; biomarkers; gene-environment interaction; neurotoxicity

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

The major goals or specific aims (SA) of this project are to:

SA-1: Use blood-borne extracellular vesicles (EVs) originating from the central nervous system (CNS) of PD patients to elucidate their brain exposome and metallome; and determine whether individual toxicants or mixtures, independently or synergistically with LRRK2 activation status, represent predictive biomarkers of disease. **Months: 1-24:** 40% of completion.

SA-2: Identify gene-environment (GxE) interactions and potential therapeutic strategies relevant to PD-linked DA neurodegeneration and LRRK2 pathology. **Months: 1-36:** 30% of completion.

SA-3: Determine if mice chronically exposed to the neurotoxicant profile with the strongest link to LRRK2 activation (in SA-1) recapitulate clinical and pathological signs of PD. **Months: 15-36.** Year 2 start.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1) Major activities:

Since the start of the project in September 2022, our major activities have been staffing, sample acquisition, reagent ordering, and kick start of the different specific aims as described in more details below. First, Drs. Re and Miller hired new personnel to assemble the teams necessary to carry the project in both laboratories. Dr. Re hired a technician and a talented staff associate, instead of a post-doc, due to lack of candidates with the required expertise. The personnel hired by Dr. Re both have expertise in neurotoxicology, neurodegenerative diseases and extracellular vesicle-based biomarker research. Dr. Miller identified an excellent post-doc candidate with expertise in neurotoxicology, but due to visa issues the start date was pushed to May 2023. Then, as described in the statement of work we obtained patient samples from the Columbia Department of Neurology Biobank: healthy controls (n=35); sporadic PD (n=35); unaffected carriers LRRK2 G2019S+/PD- (n=8 instead of 35 initially planned as samples run out for other projects); affected LRRK2 G2019S+/PD+ (n=35).

We also obtained 60 samples from Biomedical Research Foundation Academy of Athens: healthy controls (n=30); sporadic PD (n=30). Drs. Re and Miller are currently in contact with several international collaborators to obtain more unaffected carriers LRRK2 G2019S+/PD- to compensate the samples that could not be provided by Neurology.
Our next major activities have been ordering, validation of reagents, optimization of protocols, amplification of the patient cells obtained from NINDS, and processing of human samples for the biomarker study.

2) Specific objectives:

SA-1: Use blood-borne EVs originating from the CNS of PD patients to elucidate their brain exposome and PD-linked metallome and determine whether individual toxicants or mixtures, independently or synergistically with LRRK2 activation status, represent predictive biomarkers of disease.

Timeline: Months 1-24

Major Task 1: Isolate CNS neuronal (CNTN2) and astrocyte (GLAST) EVs from human control, PD-affected and unaffected LRRK2 carrier plasma samples. Months 1-11.

Key outcomes:

To ensure rigor and reproducibility in our studies, before ordering in bulk enough CNTN2 and GLAST antibodies to isolate all the neuronal and astrocyte EVs planned to be analyzed in this project (from ~600 patient plasma samples), we requested to receive from each manufacturer, several lots of antibodies available in large quantities and we carried out a series of validation experiments for each of these reagents. We were able to confirm that several lots of the GLAST antibody provided can successfully isolate astrocyte-derived EVs, enriched as expected in pan EV markers (CD81, CD9, CD65, syntenin) and in astrocyte-specific proteins (GFAP, GLUL). However, none of the CNTN2 lots received provided EV and neuronal marker (NFL, SYP) enrichment data that were as satisfactory as in our pilot study. In fact, the data obtained showed a quite poor isolation efficiency ($1/10^{th}$ of the GLAST-EV isolation efficiency). Therefore, we decided to explore the possibility to use an alternative membrane surface marker for neuronal EVs, we tried L1CAM, NCAM, CD90 and CD56 but none of them yield to a satisfactory isolation of EVs enriched in neuronal markers. Because next to astrocytes, microglial cells are also critical in PD pathogenesis, we tried to develop a protocol to isolate EVs of microglial origin. We tried several antibodies targeting different microglia-specific membrane surface markers (TMEM119, CD11b, and P2Y12) and were finally successful to efficiently isolate EVs enriched in pan EV markers and one microglia marker (P2Y12) with an antibody against P2Y12. Although, we could not find (so far) in P2Y12-EVs enrichment in other classical microglial markers, to decide which types of EVs we should isolate from our patient samples, we decided to compare LRRK2 levels in GLAST-, CNTN2- and P2Y12-EVs. The data obtained are illustrated in **Figure 1**. These ELISAs, performed by a company specialized in ultrasensitive LRRK2 and PD assay development, demonstrate that astrocyte-derived GLAST-EVs are by far the most enriched in total LRRK2 protein and thus certainly the most appropriate EVs to determine differences in LRRK2 quantity, phosphorylation state or kinase activity in our different control and PD patient samples. We thus decided to proceed with GLAST-EV isolation and thus astrocyte-derived EVs for our study. We have now completed the isolation of GLAST-EVs from our samples for LRRK2 analysis and metal analysis (**subtasks 2 and 3, respectively**). The isolation of EVs for the exposomics (**subtask 1**) was delayed by the late hiring of Dr. Miller's post-doc (in May 2023) however it was necessary to really identify a highly qualified candidate for this extremely innovative part of the study. Since her start, Dr. Miller's post-doc has made significant contribution to the project.

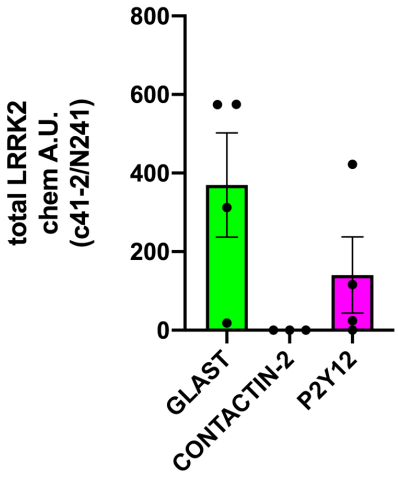

Figure 1: Relative total LRRK2 levels measured by ELISA in GLAST-, **Contactin-2-, and P2Y12 EVs.**

In particular, she has optimized our EV isolation method to reduce background organic toxicant contaminations to trace levels. She also greatly improved the organic extraction method to substantially decrease the detection limits of the high-resolution mass spectrometry (HRMS).

While the Miller's lab was carrying out these crucial experimental optimizations, a new neuronal-specific membrane surface marker, ATP1A3, has been reported by the group of Dr. Ikezu (Mayo Clinic, Florida) to enable the efficient and reproducible isolation of EVs from neuronal origin. We corresponded with Dr. Ikezu and are now testing this antibody targeting ATP1A3, and we should know in the coming weeks whether it can successfully isolate EVs enriched in both pan EV and neuronal-specific markers. We will also verify the LRRK2 and metal content of ATP1A3-neuronal EVs before deciding to include them in our study. For this reason, we have for now put on hold **Subtasks 1** of **Major Tasks 2, 3 and 4** (processing of the EV samples for exposomics, metallomics and LRRK2 status analyses) that were supposed to start on Month 8-10 of the award. If we confirm that ATP1A3 EVs are like GLAST-EVs enriched in LRRK2 and metals, we will isolate ATP1A3 EVs from the same samples that we used for GLAST-EVs and combine them together before downstream analyses. We anticipate that this minor delay will be highly valuable if ATP1A3-EVs finally allow us to meet our original objective to investigate both neuronal and astrocyte-derived EVs.

SA- 2. Identify gene-environment (GxE) interactions and potential therapeutic strategies relevant to PD-linked dopaminergic neurodegeneration and LRRK2 pathology.
Timeline: Months 1-36
Major Task 1: Assess PD-linked metals and organic toxicants-induced neuronal death individually and as mixtures in iPSC-DA co-cultures derived from human control, PD-affected and unaffected LRRK2 carriers. Months 1-24.

- **Key outcomes:**

At the start of the award, we acquired from the NINDS Human Cell and Data Repository 5 human induced pluripotent stem cell lines (iPSC) lines, as well as one additional line from the Columbia Stem Cell Core (see **Table 1**): three lines were derived from Parkinson disease's patients carrying a mutation in LRRK2 (R1441C) and three lines were produced from age and sex-matched control patients. We successfully amplified all these human iPSC by culturing them in mTSER media on matrigel to generate stocks that should be sufficient if we ever need to go back to the first passages of the original cells during the reminder of our in vitro studies. We produced about 30-40 frozen vials for each iPSC line that are safely stored in our liquid nitrogen tank.

ND41865	Control
ND35371*D	PD patient/LRRK2 (ARG1441CYS variant)
ND50054	PD patient/LRRK2 (ARG1441CYS variant)
NH50351	control

Table 1: Induced pluripotent Stem Cell (iPSC) lines selected for the study.

We then differentiated our 6 iPSC lines in human neural precursors (NPC) that are easier to culture and amplify than iPSCs and represent an intermediate step toward our goal of generating ventral midbrain cultures. Figure 2 shows the characterization of the NPCs obtained with one control line (Figure 2A-D) and one Parkinson patient-derived line (Figure 2E-H). We followed the well-established protocol from Reinhardt et al (PLoS One 2013). In brief, once iPSC colonies reached 40-50% confluence, the media was changed to iPSC media without FGF2 (to deactivate the pluripotency signals), and CHIRR (3 μM), Purmorphamine (500nM), SB (10 μM) and Dorsomorphin (1 μM) were added to mimic the induction of the neural tube. The latter two factors were then replaced with ascorbic acid (AA, 150 μM). As shown, we were able to confirm that we successfully obtained pure NPC cultures as demonstrated by strong Nestin/Mushashi double immunostaining. We produced about 20 frozen vials for each NPC line that are now safely stored in our liquid nitrogen tank.

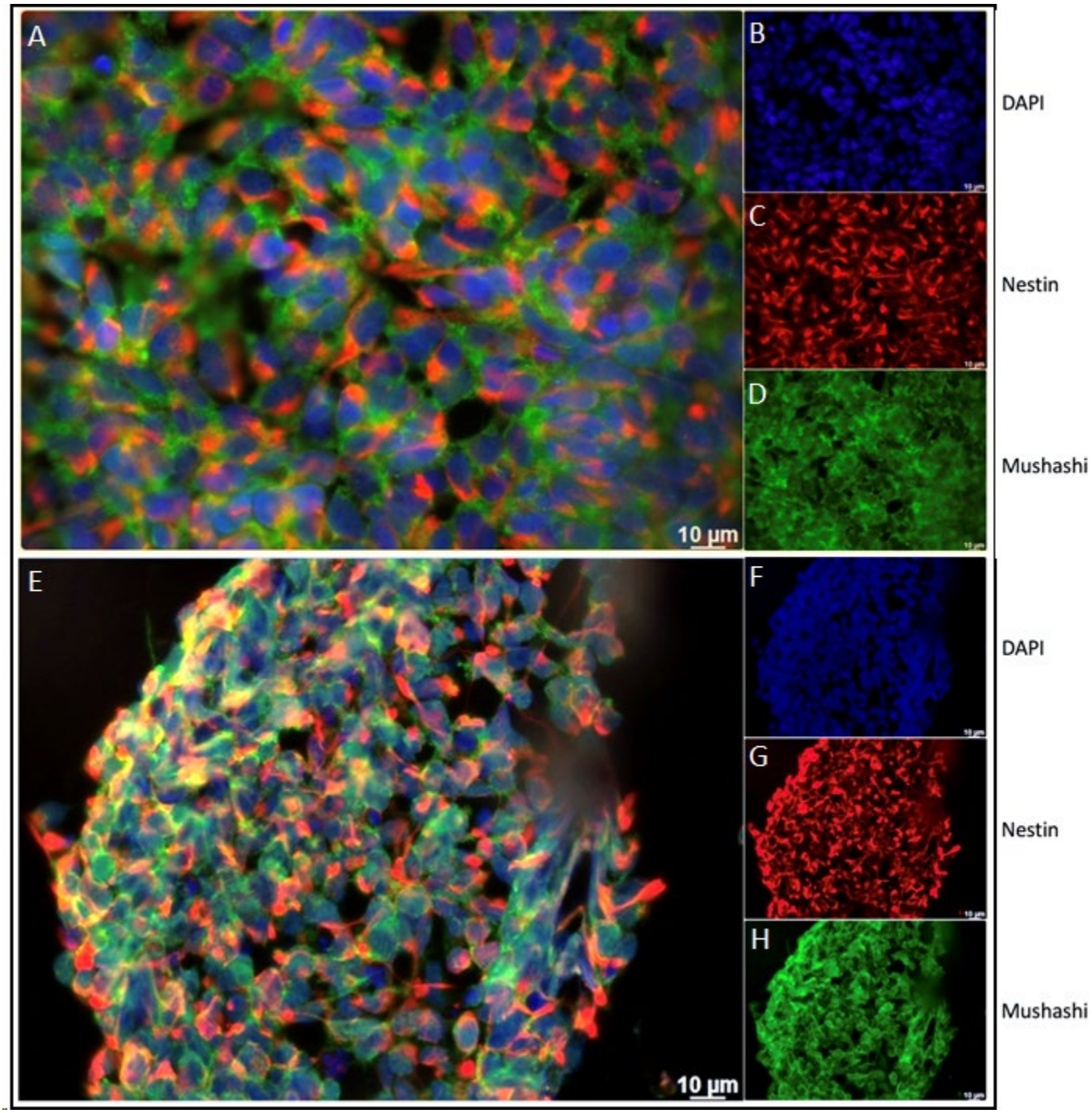

Figure 2. Characterization of patient-derived and healthy donor human neural precursors (NPCs). Labeling and immunostaining of: (**A**) healthy donor control NPC line or (**E**) patient-derived NPC line (**B, F**) show nuclei with DAPI, (**C, G**) intermediate filament protein of neural progenitor cells (Nestin) and (**D, H**) marker for proliferating neural precursor cells (Musashi-1).

We also successfully differentiated human NPCs towards a ventral midbrain dopaminergic lineage following a 45-day differentiation protocol (Barbuti et al., Sci Rep. 2021 Nov 9;11(1):21946). **Figure 3** illustrates the characterization of these cultures that are enriched in dopaminergic neurons as attested by immunopositivity for the rate-limiting enzyme of the biosynthesis of dopamine, Tyrosine Hydroxylase (TH) colocalized in some neurons with the pan neuronal marker (Tuj1) and the midbrain marker FoxA2. Briefly, at the beginning of the differentiation N2B27 base media with Purmorphamine (1µM), AA (150 µM) and FGF8b (100ng/mL) was added. At day 10 (d10-45), these factors were changed with AA (200 µM), cAMP (500 µM), BDNF (10ng/mL), GDNF (10ng/mL), TGFß3 (1ng/mL) and DAPT (1 µM) until differentiation was complete.

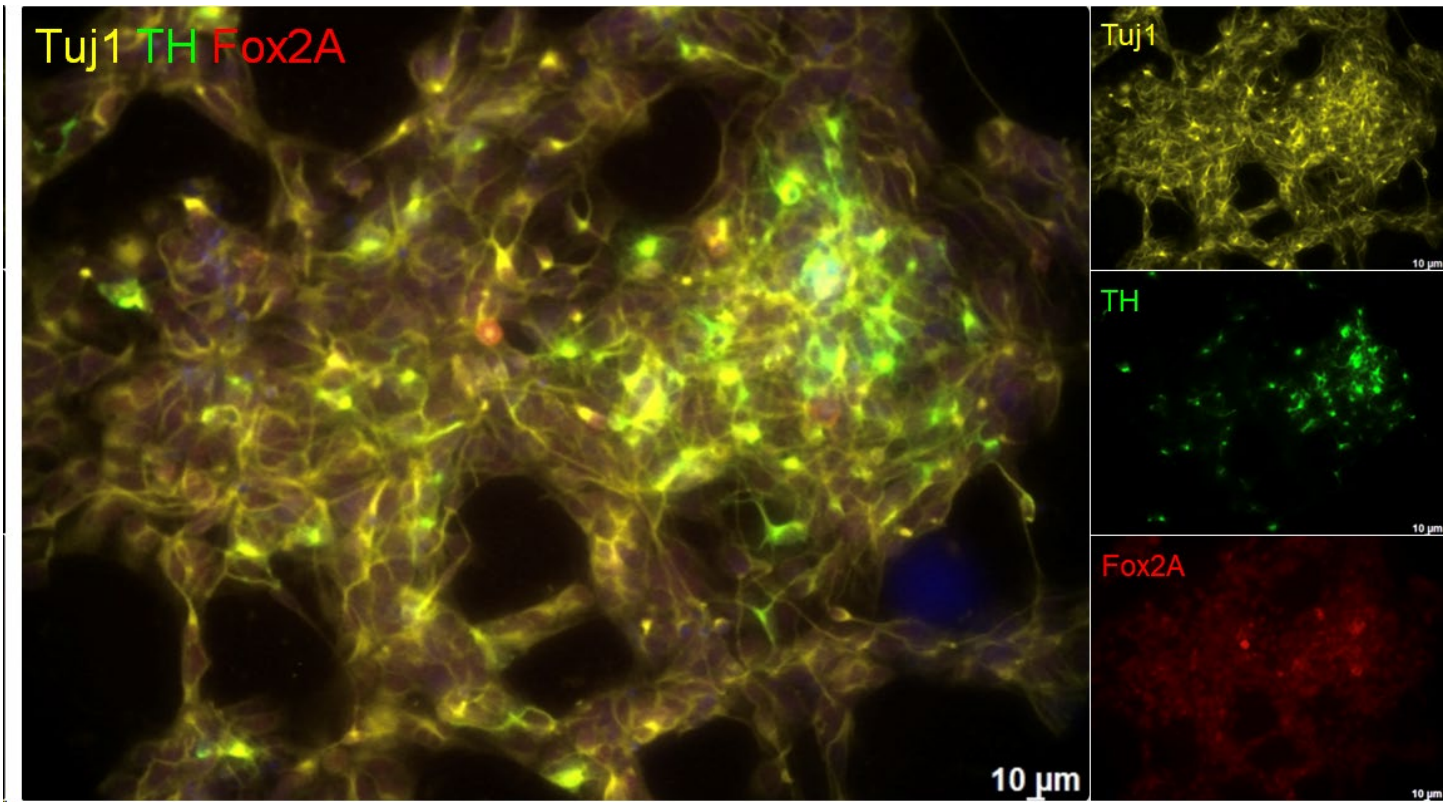

Figure 3. Characterization of patient-derived midbrain dopaminergic cultures. Immunostaining for the pan neuronal marker Tuj1, dopaminergic marker TH and the midbrain marker FoxA2.

We started to screen in these human ventral midbrain cultures the neurotoxicity of different metals and organic toxicants individually (**Subtask 1**). We initially worked in 48 well plates but we are currently working on adapting our in vitro system to 96 well plates to allow the testing of multiple conditions and replicates on the same plate and decrease the density of neurons present to an amount more amenable to automatic counting.

We started with 3 DA cell lines: 2 control lines and 1 Parkinson patient derived. After plating, iPSC-neurons were evaluated longitudinally (days 7, 14, and 21) for chronic toxicity of individual metals (Mn, As, Fe, Cu and Al) and organic toxicants (organophosphate chlorpyrifos, the pyrethroid cis-permethrin, and the PCB mixture aroclor). We are in the middle of the lengthy manual counting of TH-positive versus total (Tuj1-positive) neurons in these plates, as the neuronal cultures are too dense after 21 days for an automatic system. However, these experiments will provide us with critical information on neurotoxicity doses and kinetics while we are working on adapting the cultures to a higher throughput capacity.

In parallel, to inform our metal EV biomarker and gene-environment interaction studies, we decided to start a bit in advance some experiments planned in **Major task 2** (24 months of the award), including the measure of metals in EVs isolated from our human cultures. For this, we differentiated some control and PD human NPCs into astrocyte cultures and test the same metals as in the neurotoxicity screen (Mn, As, Fe, Cu and Al) to determine which metals are found to be extruded by astrocytes in EVs, 3 and 7 days after 2.5 µM of these metals were applied to these cultures. The data obtained are summarized in **Figure 4**. First, we confirmed that our NPC cultures successfully differentiated into pure astrocyte cultures immunopositive for GFAP and exhibiting the classical stellate shape if astroglial cels (**Figure 4A**). EVs were isolated from astrocyte culture medium using the precipitation kit Exoquick (SystemBiosciences). EV preparations were characterized by TEM (**Figure 4B**) and found to exhibit sizes ranging from 30-200 nm, as well as the expected morphology and shape (round or cup shaped) and with an electron-dense double membrane, We also validated that they express pan EV markers (CD81, shown in **Figure 4C**; but also syntenin, not shown) as well as astrocyte specific markers (GFAP and GLUL).

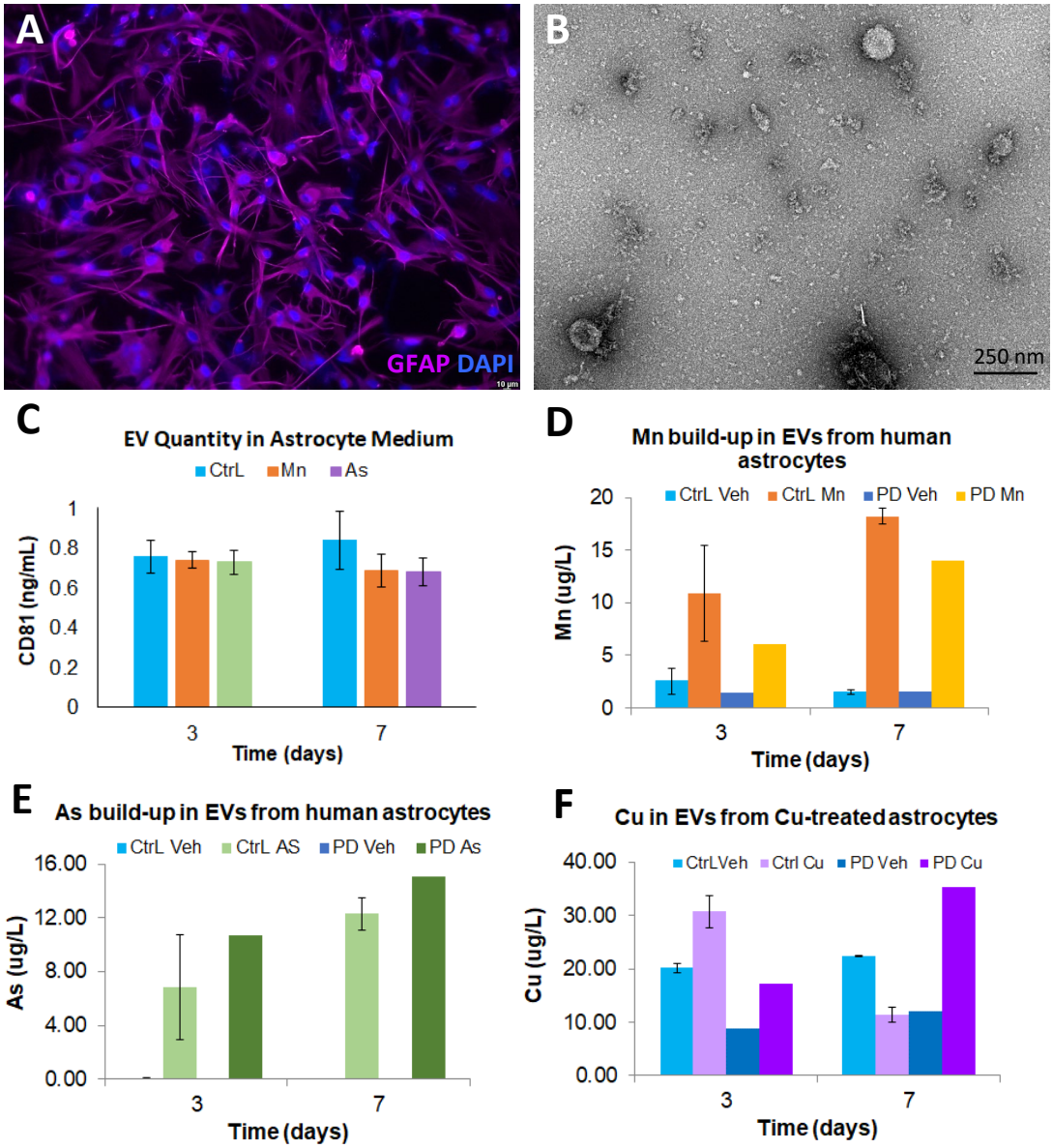

Figure 4. Release of metals via the EV pathway in human control and PD astrocyte cultures. (A) Efficient differentiation of our human iPSC/NPC lines into pure astrocyte cultures as demonstrated by immunostaining for the astrocyte marker glial fibrillary acidic protein (GFAP) and DAPI nuclear staining. (**B**) Transmission electron microscopy (TEM) characterization of the EVs released by human astrocytes confirms expected nanoscale sizes, appearances and shapes and thus the quality of our EV preparation. (**C**) EV quantity measurement by ELISA determination of the pan EV marker CD81 in the medium of control cultures and of astrocytes treated with either 2.5 µM of Mn or As. (**D**) Mn levels determined by inductively coupled plasma mass spectrometry (ICP-MS) in EVs isolated from control (CtrL) and Parkinson's patients (PD) astrocytes, treated with vehicle (Veh; water) or Mn ($MnCl_2$). (**E**) As levels determined ICP-MS in EVs isolated from CtrL and PD astrocytes, treated with Veh or As (H_3AsO_4). (**F**) Cu levels determined ICP-MS in EVs isolated from CtrL and PD astrocytes, treated with Veh or Cu ($CuCl_2$).

As shown in Figure 4C, we found that the quantity of EVs released by control cultures and cultures treated with metals (Mn and As shown here) were the same over 7 days. Though. in Figure 4D, we see that the levels of Mn in EVs from astrocytes treated with 2.5 µM Mn significantly build up as compared to vehicle (Veh) conditions. When we compare EV isolated from astrocyte cultures produced from healthy control donors (CtrL) and astrocytes produced from PD patients (PD), it appears that PD patient astrocytes maybe slightly less efficient in extruding Mn through the EV pathway. However, for As we observe the inverse trend as EVs from PD astrocytes appear to be loaded with higher levels of As (Figure 4E). As expected, we also observed that Mn which is an

And logically, As which is a rare xenobiotic metal is only detected in the EVs of cultures exposed to As. Then, when we exposed astrocytes cultures to Cu which is an abundant endogenous element (Figure 4F), we found that both control donor and PD astrocytes could eliminate this metal via the EV pathway after 3 days, however at day 7, only the PD astrocytes appeared to still be in need to eliminate the metal via EVs. Another interesting observation is that Cu was quite abundant in EVs from Veh control cultures, but its levels appear much lower in EVs isolated from Veh PD astrocytes. We performed the same experiments with Fe and Al but we found that these two elements were found at even higher levels in EVs from Veh cultures (80 µg/L for Al and 40 µg/L for Fe) and the addition of 2.5 µM of additional metals did not appear to change anything. For these ubiquitous and more abundant metals, we anticipate that we will need to expose cells to much higher levels before being able to observe whether brain cells can use the EV pathway to eliminate these metals too.
Although, all these results will need to be confirmed with a higher number of human cell lines (for now n=3) and in a higher number of replicates (also n=3), we observed some intriguing differences in the way different metals are eliminated via the EV pathway and also regarding their relative abundance in EVs even in control conditions. The observation that astrocytes carrying a LRRK2 mutation appear to handle metal release via the EV pathway differently as compared to control donor lines suggest some fascinating GxE interaction that we will keep investigating in more details during the next 2 years of the project.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Dr. Carolina Duarte-Hospital was able to participate in the 2023 Exposome Bootcamp at Columbia University. The provided training on the high-resolution mass spectrometry workflows that she will use for this project.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Dr. Miller attended the National Academy of Sciences Committee on Toxicology in September, 2023. This committee is sponsored by the Department of Defense and he had discussions with several key stakeholders including discussion of this project. Dr. Miller has been working with leadership at the Michael J. Fox Foundation as they develop their program on environmental factors in Parkinson's disease. The work on this project can inform future efforts in the field. Dr. Miller was also asked by the National Institute of Neurological Diseases and Stroke to participate in their advisory board on the Neural Exposome.

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

SA-1: Use blood-borne EVs originating from the CNS of PD patients to elucidate their brain exposome and PD-linked metallome and determine whether individual toxicants or mixtures, independently or synergistically with LRRK2 activation status, represent predictive biomarkers of disease. Timeline: Months 1-24
We plan to fully complete SA-1 over the second year of the project. Once we confirmed whether ATP1A3-EVs are enriched in LRRK2, we will complete the final EV isolation from the patient samples. The Trace metal core, the LRRK2 specialists and the Exposomics core led by Dr. Miller have optimized protocols and are ready to analyze the samples. Our epidemiologist and clinical collaborators will help with the final analysis.
SA- 2. Identify gene-environment (GxE) interactions and potential therapeutic strategies relevant to PD-linked dopaminergic neurodegeneration and LRRK2 pathology. Timeline: Months 1-36
We anticipate bringing SA-2 to 60% of completion as planned. We will complete the analysis of individual metals and organic toxicant groups, to be ready in year 3 to investigate the mixtures identified in SA-1.
Specific Aim 3. Determine whether mice chronically exposed to the neurotoxicant profile with the strongest impact on LRRK2 pathology and disease progression recapitulate clinical and pathological signs of PD. Timeline: Months 15-36
We will start our animal study as soon as we have identified the neurotoxicant mixture to chronically administer to animals. We anticipate completing the 6 month-exposure study before the end of year 2. The pathological analyses will be performed, as planned on year 3.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to report

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

As noted above, our findings will be communicated to key stakeholders in the Parkinson's disease community. Our work will help explain the complex gene-environment contributors to the disease.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

As described in "Accomplishments" the only minor change in approach is that instead of using antibodies targeting the neuronal membrane surface marker "contactin-2" to isolate EVs of neuronal origin, we plan on using the recently discovered marker "ATP1A3" because contactin-2 antibodies have shown a poor level of reproducibility in their efficiency to isolate neuronal-specific EVs. However, we still need to fully validate this new target in our laboratory. If it fails to meet our rigor and reproducibility criteria, we will solely focus on EVs from astrocyte which are in any case the most abundant cells in the brain and critical players in PD and in metal and organic toxicant neurotoxicity.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

The major delay we have encountered concerns the hiring of post-doctoral researchers. There is currently a quite alarming national shortage of post-doc applicants that Drs. Re and Miller have both experienced. After several months of struggle to find a single qualified post-doc applicant, Dr. Re decided to hire instead a more junior but highly qualified staff associate who previously did her Master thesis with her. However, for the exposomics which is the most innovative and sophisticated part of the study, Dr. Miller really had to take the time to find a highly experienced candidates who was only able to start in his lab in May 2023 after resolving visa issues. Since she started, Dr. Miller's post-doc has made significant contribution to the project and has optimized all the protocols to now run the samples in the shortest possible time. Another delay pertains to our decision to try a new very recently described membrane surface marker to isolate neuronal EVs. We anticipate that this minor delay will be highly valuable if ATP1A3-EVs finally allow us to meet our original objective to investigate both neuronal and astrocyte-derived EVs.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Delays in hiring staff had a significant impact on expenditures and we plan on hiring additional staff to speed up the project as soon as we assess it becomes necessary. Also, the spending of the funds for metallomics, exposomics and LRRK2 analyses has been delayed until we resolve whether we will add ATP1A3-EVs to the already isolated GLAST-EVs. Since the writing of the proposal many reagents and service costs have significantly increased but we are working on bulk purchase and other strategies (e.g., digesting ourselves the samples for metal analysis) to meet the original budgeted costs.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Not applicable.

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

Not applicable.

Significant changes in use of biohazards and/or select agents

Not applicable.

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

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

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

Nothing to report.

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

  Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as*

noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

Nothing to report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Gary Miller
Project Role:	Partnering PI
Researcher Identifier (e.g. ORCID ID):	0000-0001-8984-1284
Nearest person month worked:	1.0 CM
Contribution to Project:	Dr. Miller's role is to contribute to the design, organization, and supervision of all the experiments described in this proposal, and review all the work performed by the postdoctoral fellow involved in this project. With a focus on mass spectrometry. He will also analyze, interpret, report on, and disseminate all the generated results.
Funding Support:	*(Complete only if the funding support is provided from other than this award).*

Name:	Carolina Duarte-Hosptial, Ph.D.
Project Role:	Postdoctoral Associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3.5 CM
Contribution to Project:	Dr. Duarte-Hospital has been focused on developing the mass spectrometry workflow for the extracellular vesicles. She has worked with several of the mass spec core team members and investigators in Dr. Re's lab.
Funding Support:	*(Complete only if the funding support is provided from other than this award).*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Previous Active Grant that has Closed

Title: Mega-scale Identification tools for xenobiotic metabolism (5U2C ES030163-02)
Time Commitments: 0.58 calendar months
Supporting Agency: National Institute of Environmental Health Sciences
Name and Address of Grant Officer:
David M. Balshaw
P.O. Box 12233
Durham, NC USA 27709
Performance Period: 09/01/18-06/30/22
Level of funding:
Project Goals: This study is to develop and test new chemical identification tools to identify hundreds of thousands of foreign chemical metabolites.
Specific Aims: Aim 1: Develop xenobiotic metabolite prediction tools and database to support high-throughput xenobiotic metabolite identification. Aim 2: Develop targeted multi-well biotransformation tools using subcellular fractions and cells with targeted expression of xenobiotic transformation enzymes for use with ultra-high-resolution mass spectrometry and targeted ion dissociation MS/MS for automated high-throughput xenobiotic metabolite identification. Aim 3: Support real-time testing of xenobiotic metabolite compound identification tools to identify environmental chemical metabolites for NIH-supported research programs. Aim 4: Participate in SEPCC activities and promotion of metabolomics development and outreach, including research presentations and publications, symposia and workshops, development of best practices, training and professional development, and community engagement.
Overlap: No scientific or budgetary overlap

Title: Dissecting the pathogenesis and outcomes of PSC using multi-omics by studying the exposome and genome (5RC2 **DK118619-04)**
Time Commitments: 1.2 calendar months
Supporting Agency: National Institute of Diabetes and Digestive and Kidney Diseases
Name and Address of Grant Officer:
Averell H. Sherker
31 Center Dr.
Bethesda, MD 20892
Performance Period: 09/01/18-07/31/23
Level of funding:
Project Goals: The overall aim of this project is to utilize a multi-omic approach to delineate the role of the exposome in primary PSC.
Specific Aims: Using a suite of bioinformatic tools and available genetic variation data, we aim to discover stable, detectable, omics-based disease signatures in blood (Aim 1) and stool (Aim 2) that when integrated with clinical data (Aim 3) will reveal biological pathways driving disease pathogenesis and outcomes.
Overlap: No scientific or budgetary overlap

Title: Synaptic Vesicle Glycoprotein 2C (SV2C) and Psychostimulant Actions (U18 DA052498)
Time Commitments: 0.6 calendar months
Supporting Agency: National Institute on Drug Abuse
Name and Address of Grant Officer:
Christopher Conrad
3WFN
301 North Stonestreet Ave
Bethesda MD 20892
Performance Period: 09/30/2020 – 09/29/2021
Level of Funding:
Project Goal: New approaches to treat methamphetamine addiction are desperately needed. Enhanced synaptic transmission of dopamine is a key feature of methamphetamine action. We have identified a novel mediator of dopamine release (SV2C) that we propose will reduce the reinforcing properties of methamphetamine. Given the observed effect of SV2C loss on dopamine transmission and its restricted expression pattern we hypothesize that SV2C, through its ability to retain dopamine within synaptic vesicles, regulates the psychostimulant effects of methamphetamine, and therefore is a druggable target for psychostimulant substance use disorder. This project will establish a novel *in vitro* assay to measure SV2C activity and identify compounds that regulate SV2C activity.
Specific Aims: The experiments proposed here will establish a novel in vitro assay to measure SV2C activity (Aim 1) and identify compounds that regulate SV2C activity (Aim 2).
Overlap: No scientific or budgetary overlap

New Active Projects

Title: Novel exposomics and extracellular vesicle biomarkers to unravel gene-environment interactions and mechanisms of neurodegeneration in Parkinson's disease. (W81XWH2210628)
Time Commitments: 1.00 calendar months
Supporting Agency: Department of Defense
Name and Address of Grant Officer: Daniel M. Monson
Congressionally Directed Medical Research Program Office
Performance Period: 09/01/2022-08/31/2025
Level of Funding:
Project Goal: Our objectives are to determine whether exposure to, and accumulating levels of organic toxicants, metals or their mixtures, interact with silent genetic factors, such as mutations in the gene encoding LRRK2, to modulate the risk of developing PD. We will address this question using novel cellular and in vivo

preclinical models, as well as state-of the-art brain biomarkers, analytical and “omics” techniques in two independent PD clinical cohorts.
Specific Aims:
Overlap: No scientific or budgetary overlap

Title: Vesicular modulation of dopamine neuron toxicity (3R01ES023839-07S1)
Time Commitments: 0.12 calendar months
Supporting Agency: National Institute of Environmental Health Sciences
Name and Address of Grant Officer:
Jonathan Hollander
P.O. Box 12233
Durham, NC USA 27709
Performance Period: 06/20/2022 - 12/31/2023
Level of funding:
Project Goals: Environmental toxicant exposure is a risk factor for neurodegenerative disease. This supplement expands the Parkinson’s disease-related research of the parent grant to examine environmental drivers of Alzheimer’s disease and related dementias using similar models and approaches.
Specific Aims: Aim 1: Characterize AD-associated behavioral and pathologic outcomes in *C. elegans* exposed to putative cognition-impairing toxicants. Specific Aim 2: Discover metabolomic pathways altered by putative cognition-impairing toxicants.
Overlap: No scientific or budgetary overlap

Title: Epidemiological Integration of Genetic Variants and Metabolomics Profiles in Washington Heights Columbia Aging Project (3RF1 AG066107-S1)
Time Commitments: 1 calendar months
Supporting Agency: National Institute of Aging
Name and Address of Grant Officer:
Alison Yao
Building 31, Room 5C27
31 Center Drive, MSC 2292
Bethesda, MD 20892
Performance Period: 09/01/2022 – 08/31/2024
Level of funding:
Project Goals: EXCEL AD will advance the science of exposomics for AD/ADRD. In addition to improving discovery on the parent award, we will also help develop a community dashboard to facilitate exposure studies, protocol development and data sharing at other institutions.
Specific Aims: Aim 1. To improve capacity, throughput, and identification of exogenous and endogenous chemical features through expansion of our high-resolution mass spectrometry capabilities. Aim 2. To perform pilot studies for ADRC investigators using the untargeted exposomics and metabolomics platform. Aim 3. To develop, optimize, and distribute workflows via an online EXCEL AD Community Dashboard among other ADRCs and AD research groups interested in incorporating exposomics into their studies.
Overlap: No scientific or budgetary overlap

Title: Environmental Exposure Assessment Research Infrastructure Preparatory Phase Project (101079789)
Time Commitments: 0.12 calendar months
Supporting Agency: European Research Executive Agency (REA)
Name and Address of Grant Officer:
Shachar D'vir
Kamenice 753/5 | 625 00 Brno | Czech Republic
Performance Period: 10/01/2022-09/30/2025
Level of funding:

Project Goals: EIRENE is an infrastructure grant to support exposomics. The team will be working on standard operating procedures and harmonization across laboratories. Dr. Miller leads the Columbia University site.
Specific Aims:
Overlap: No scientific or budgetary overlap

Title: Exposome Therapeutics: Predictive algorithms for the environmental component of precision medicine (ALCE CU22-3434)
Time Commitments: 0.06 calendar months
Supporting Agency: AlleyCorp Enterprises, LLC
Name and Address of Grant Officer:
Kevin Ryan
31 West 52nd Street
New York, NY 10019
Performance Period: 06/30/2022-06/29/2024
Level of funding:
Project Goals: Advance chemistry for SV2C drug design. Create priority list of exposomic drug targets.
Specific Aims:
Overlap: No scientific or budgetary overlap

Title: Characterizing the link between multiple environmental exposures and Parkinson's disease exacerbation (R01 ES034373)
Time Commitments: 0.6 calendar months
Supporting Agency: National Institute of Environmental Health Sciences
Name and Address of Grant Officer:
Kimberly Gray
BG 530 Davis Dr. Keystone Building Rm 3070
530 Davis Dr
Durham, NC 27560
Performance Period: 09/20/2022 – 07/31/2027
Level of funding:
Project Goals: This project will leverage data on Medicare enrollees Parts A, B, and D, air pollution (PM2.5, NO2, and ozone), PM2.5 major components, weather variables, greenness, and multiple ZIP code level Census factors to estimate the link between individual and simultaneous exposure to air pollution, PM2.5 major components, weather and greenness and Parkinson's Disease (PD) exacerbation of symptoms. We will develop and apply new and existing methods to characterize a complex set of possible effect modifiers and identify multiple risk and protective factors that determine vulnerability in PD, and develop open-source R packages, promoting full transparency and reproducibility of findings across different study populations. This project will provide the foundation to inform actionable policy to help slow the PD disease burden and improve the quality of life for millions of Americans.
Specific Aims:
Overlap: No scientific or budgetary overlap

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Organization name: Biomedical Research Foundation (BRFAA) of the Academy of Athens
Location of Organization: Athens, Greece
Partner's contribution to the project: Provided human plasma samples for the study.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.