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CONTRACTING ORGANIZATION: Washington University, St Louis, MO

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14. ABSTRACT
Parkinson disease (PD) is characterized by accumulation of protein aggregates composed of the protein alpha-synuclein (aSyn) in structures termed Lewy bodies (LB) and Lewy neurites (LN) in multiple brain regions. Although motor symptoms are often the most visible features of the illness and are thought to relate to abnormalities in nigro-striatal dopaminergic signaling, PD also causes dementia with prominent cognitive and psychiatric symptoms in many patients. The molecular and circuitry mechanisms that lead to PD dementia are not clear, and there are no effective treatments to prevent or slow progression of dementia in PD. Multiple studies indicate that pathological aSyn aggregation plays a key role in PD, and a growing number of reports now demonstrate that aSyn spreading can be modeled in vivo by stereotaxic injection of aSyn preformed fibrils (PFFs) into the striatum where it then spreads in a time-dependent manner to multiple connected regions including the substantia nigra as well as the cortex and amygdala. Genetic association studies implicate the apolipoprotein E (APOE) genotype as a strong risk factor for dementia in PD. Our preliminary data indicates that apoE isoforms directly regulate aSyn spreading within the nigro-striatal circuit, but whether APOE genotype affects aSyn spreading in neocortical and limbic regions, and whether this contributes to deficits in cognitive behavior and disruption of function connectivity in brain networks is unknown.

15. SUBJECT TERMS
Parkinson disease, Parkinson disease dementia, Lewy body dementia, alpha-synuclein, apolipoprotein E, pre-formed fibril, cortex, amygdala, striatum, cognition, functional connectivity

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TABLE OF CONTENTS

1. INTRODUCTION:

Parkinson disease (PD) is characterized by accumulation of protein aggregates composed of the protein alpha-synuclein (aSyn) in structures termed Lewy bodies (LB) and Lewy neurites (LN) in multiple brain regions. Although motor symptoms are often the most visible features of the illness and are thought to relate to abnormalities in nigro-striatal dopaminergic signaling, PD also causes dementia with prominent cognitive and psychiatric symptoms in many patients. The molecular and circuitry mechanisms that lead to PD dementia are not clear, and there are no effective treatments to prevent or slow progression of dementia in PD. Multiple studies indicate that pathological aSyn aggregation plays a key role in PD, and a growing number of reports now demonstrate that aSyn spreading can be modeled *in vivo* by stereotaxic injection of aSyn pre-formed fibrils (PFFs) into the striatum where it then spreads in a time-dependent manner to multiple connected regions including the substantia nigra as well as the cortex and amygdala. Genetic association studies implicate the apolipoprotein E (*APOE*) genotype as a strong risk factor for dementia in PD. We recently published that apoE isoforms directly regulate aSyn spreading within the nigro-striatal circuit, but whether *APOE* genotype affects aSyn spreading in neocortical and limbic regions, and whether this contributes to deficits in cognitive behavior and disruption of functional connectivity (FC) in brain networks is unknown. The purpose of this project is therefore to test the hypothesis that *APOE* genotype regulates spreading of aSyn pathology to cortical and limbic regions, negatively affecting functional connectivity and behavior related to these regions.

2. KEYWORDS:

Parkinson disease (PD), Parkinson disease dementia (PDD), dementia with Lewy bodies (DLB), alpha-synuclein (aSyn), apolipoprotein E (APOE), pre-formed fibril (PFF), cortex, amygdala, striatum, cognition, functional connectivity (FC), functional connectivity optic intrinsic signaling (fcOIS).

3. **ACCOMPLISHMENTS:**
   - **What were the major goals of the project?**

Specific Aim 1	Timeline	% Complete
Major Task 1: Generate mice and perform aSyn pre-formed fibril (PFF) Injections	Months	
Validate efficacy of aSyn PFFs in wildtype mice, including controls with aSyn monomer in wildtype mice and aSyn PFFs in aSyn knockout mice	1-3	100
Breed Apoe KO and APOE2, APOE3, and APOE4 mice	1-6	100
Inject cohorts of mice with aSyn PFFs	6-9	100
Local IACUC Approval (amendment needed for collaboration with Dr. Bauer for fcOIS)	1	100
Major Task 2: Assess pathology in neocortical and limbic regions		
Perfuse mice, section brains, and perform immunohistochemistry for pSyn, GFAP, CD68	10-14	90
Quantify and map brain pathology	12-16	90
Perform statistical analysis to relate APOE genotype to brain pathology	12-16	90
Specific Aim 2		
Major Task 3: Cognitive Testing in mice injected with aSyn PFFs		
Perform cognitive test battery in Apoe KO and APOE2, APOE3, and APOE4 mice injected with aSyn PFFs	12	100
Data analysis and correlation with brain pathology	12-14	80
Specific Aim 3		
Major Task 4: fcOIS imaging in mice injected with aSyn PFFs		
Validation of functional connectivity phenotype in aSyn PFF injected mice vs aSyn monomer control	1-6	100
fcOIS imaging in Apoe KO and APOE2, APOE3, and APOE4 mice injected with aSyn PFFs	7-13	100
Data analysis and correlation with brain pathology	9-15	90

- **What was accomplished under these goals?**

1) **Major activities** during this project period included: 1) acquisition and analysis of behavior and functional connectivity data at 3 and 6 months post-injection in original cohort of young APOE knockin mice injected with aSyn PFFs, 2) injection of an additional cohort of aged APOE knockin mice with aSyn PFFs, 3) acquisition of behavior and functional connectivity data at 3 months post-injection in the additional cohort of aged APOE knockin mice injected with aSyn PFFs.

2) **Specific objectives** of this project include: 1) Investigate the effect of *APOE* genotype on aSyn spreading in cortical and limbic regions, 2) Determine the effect of *APOE* genotype on cognitive behavior in the context of pathological aSyn aggregation, and 3) Determine the impact of aSyn spreading on brain functional connectivity.

3) **Significant results** obtained during this project period include the following:

   a. **Measurement of APOE genotype effect on aSyn PFF-induced spreading of aSyn pathology in mouse cortex.** APOE is the strongest genetic risk factor for PDD and DLB. Humans express three main isoforms of APOE (APOE2, APOE3, APOE4) which differ by only 1 amino acid. Individuals homozygous for APOE4 have up to a 6-fold increased risk of PDD/DLB compared to the more common APOE3 genotype, whereas the more rare APOE2 genotype may confer some protection against developing PDD. We previously showed that APOE genotype affects progression of synucleinopathy in the brainstem and midbrain in two mouse models, but the effect of APOE genotype on aSyn pathology in the cortex remained less clear (Davis et al 2020 *Science Translational Medicine*). In this project we examined the effect of APOE genotype on spreading of aSyn pathology to the cortex, following injection of aSyn PFFs in the striatum of APOE knockin mice in which the mouse Apoe gene is replaced with one of the human APOE alleles (E2, E3, E4). In mice injected at 3-4 months of age, we observed a trend toward an increase in aSyn pathology in the right hemisphere motor cortex of APOE4 mice at 6 months post-injection, although most regions showed no significant difference across genotypes at this age and post-injection interval (Fig 1).

   b. **Measurement of APOE genotype effect on aSyn PFF-induced cognitive deficits in mice.** We evaluated cognitive behavioral phenotypes in the same APOE knockin mice injected with aSyn PFFs and aged for 6 months. We focused our analysis on behavioral tests that have been reported to be sensitive to aSyn PFF-induced pathology. We did not observe any effect of APOE genotype on Y-maze spontaneous alteration or contextual fear conditioning, but we did observe that mice injected with aSyn PFFs showed higher thresholds for flinching and vocalizing responses compared to mice injected with aSyn monomer (a negative control which does not induce aSyn pathology spreading) (Fig 2).

c. **Optimization of measurement of aSyn PFF-induced disruption of FC in living mice**. As described in our application, we partnered with Dr. Adam Bauer in the Department of Radiology at Washington University who has pioneered an approach to measuring functional connectivity (FC) in the brains of living mice. FC is defined as the coincidence in time of neurophysiological events that occur in spatially distinct regions of the brain. FC is often viewed as one metric to evaluate how the brain functions as a network to facilitate high level cognitive processes. Disruption of FC is known to occur in humans with diseases in which aSyn forms LBs and LNs throughout the brain, including PD, PDD, and DLB. Injection of aSyn PFFs in the striatum of mice induces spreading of aSyn pathology throughout many areas of the brain cortex and subcortical structures by causing the endogenous mouse aSyn protein to aggregate within neurons. This process results in neuronal injury which we hypothesized would disrupt FC. Measurement of FC in rodents can be performed using optical measurement of oxy- and deoxy-hemoglobin with a technique called functional connectivity – optic intrinsic signaling (fcOIS). Prior to this project, we were not aware of any reported measurements of changes in FC in rodents in models of aSyn aggregation. During this project we optimized measurement of functional connectivity using fcOIS in young mice (3-4 months old) injected with aSyn PFFs, including assessment of anesthesia to improve signal to noise ratio. We collected multiple fcOIS datasets for mice injected with aSyn PFFs. Our results demonstrate a reduction in FC strength within and across all brain regions following (Fig 3). This is a substantial achievement that was critical to the success of this project and will facilitate multiple additional projects in the future to examine the role of specific risk factor genes and the effect of disease-modifying therapies for PD, PDD, and DLB.

d. **Measurement of APOE genotype effect on aSyn PFF-induced reduction of FC strength.** To evaluate the effect of APOE genotype on aSyn-dependent changes in FC, we injected APOE knockin mice with aSyn PFFs at 3-4 months of age and measured fcOIS at 2 timepoints, 3 months post-injection and 6 months post-injection. We found a significant effect of APOE genotype on FC strength over time (Fig 4A). Seed-based FC matrix analysis showed the largest effects over time, across all genotypes, in long-range connections across networks, including communication between retrosplenial, somatosensory, and motor cortex regions (Fig 4B). Interestingly, we observed reductions in the most brain regions over time in APOE2 mice, including areas involved in cognitive, somatosensory, and visual processing.

e. **Stated goals not met**:
Although we were able to measure significant reductions in FC across multiple cortical regions in mice injected with aSyn PFFs, and

although we detected a significant effect of APOE genotype on FC over time, we suspected that these data may not represent the most accurate assessment of APOE genotype effect on spreading of aSyn pathology based on the lack of significant differences observed in our preliminary analysis of histopathology and cognitive behavior. Because PDD and DLB are diseases of later middle age and older humans, we hypothesized there may be a critical component of aging that we were not positioned to detect in our initial experiments. Therefore, we aged additional cohorts of APOE knockin mice to approximately 15 months and repeated the aSyn PFF injections, then aged the mice for 3 additional months post-injection and repeated the cognitive behavior battery, fcOIS imaging, and collected brains for immunohistochemistry. These datasets are currently being analyzed.

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

  This project has provided Dr. Davis with multiple opportunities for training and professional development, as outlined below:

  1) Expand knowledge of basic cellular and molecular mechanisms of neurodegenerative disorders:
     a. Dr. Davis attended weekly journal club and seminar series through the Washington University Department of Neurology, Movement Disorders Section, Division of Biological and Biomedical Sciences, and Hope Center for Neurological Disorders.
     b. Dr. Davis developed curriculum content and delivered lectures to Washington University medical students and graduate students.
     c. Dr. Davis gave two platform presentations at the 2020 American Neurological Association annual meeting on topics separate from but related to this project.
     d. Dr. Davis gave a presentation at Neurology Grand Rounds at Vanderbilt University.
     e. Dr. Davis gave a platform presentation at an international meeting focused on APOE genotype and neurodegenerative disease.
     f. Dr. Davis gave a presentation at Neurology Grand Rounds at Ohio State University.
     g. Dr. Davis gave a platform presentation at the 2023 American Neurological Association annual meeting on a separate but related topic.

  2) Gain expertise in basic scientific methods:
     a. Dr. Davis adapted methods for recombinant protein expression and purification and refined stereotaxic surgical techniques for induction of aSyn pathology in mice.

b. Dr. Davis attended regular meetings and discussions with mentors and collaborators related to basic science laboratory work.

3) Obtain formal instruction in lipoprotein biology: Dr. Davis participated in the formation of a new lipid research interest group in the Hope Center for Neurological Disorders at Washington University.

4) Continue clinical practice and gain exposure to clinical/translational research:
   a. Dr. Davis sees patients weekly in the outpatient Movement Disorders Center and attends periodically on the inpatient neurology consult service at Barnes-Jewish Hospital.
   b. Dr. Davis performed lumbar punctures and intrathecal infusions for a clinical trial for Huntington disease as well as lumbar punctures for multiple biomarker studies of neurologic disease.
   c. Dr. Davis performs study assessments, lumbar punctures, and is co-director of the Biofluid Core for the North American Prodromal Synucleinopathy (NAPS2) consortium which is studying REM sleep behavior disorder as a prodromal condition of synucleinopathies.

5) Navigate the process of transition to independent investigator: with mentoring support from committee members, Dr. Davis applied for and received a Michael J. Fox Foundation Target Advanced Program award to study the role of meningeal lymphatic vessels in the clearance of aSyn aggregates and an NIH R01 award to study the role of APOE genotype in endolysosomal processing of aSyn aggregates in glia.

- **How were the results disseminated to communities of interest?**

  Dr. Davis served as a panelist for a career development workshop for Washington University undergraduate students to increase awareness of basic and clinical research in neuroscience and neurology. Dr. Davis spoke at scientific presentations geared toward lay audiences organized by the Hope Center for Neurological Disorders at Washington University.

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

  This award has ended, but our team will continue to analyze the data collected during the award period using funds provided by Dr. Davis's institutional professional allowance, including the following:

1) Analyze behavior, functional connectivity, and immunohistochemistry data from APOE knockin mice injected with aSyn PFFs vs monomer.
2) Test the correlation between immunohistochemical measurements of aSyn pathology with behavioral phenotypes and functional connectivity.
3) Determine if any additional cohorts are needed to adequately assess potential differences between groups.

Dr. Davis will send a copy of manuscript(s) describing these analyses to U.S. Army Medical Research and Development Command when they are accepted for publication

4. **IMPACT:**
   - **What was the impact on the development of the principal discipline(s) of the project?**

The research aims proposed in this application focus on improving the understanding of the molecular mechanisms of dementia in patients with Parkinson disease (PD) which, as the leading cause of nursing home placement for PD patients, contributes to enormous societal cost and morbidity. This research has the potential for near-term impact in that it should clarify basic mechanisms of neuronal dysfunction in PD related to apolipoprotein E (APOE) genotype, the strongest genetic risk factor for PD dementia. By testing the hypothesis that APOE genotype regulates spreading of pathologic aggregates of alpha-synuclein in vulnerable regions of the forebrain, leading to neuronal network dysfunction and behavioral impairment, this project should establish a pathway from genetic risk factors to molecular neuropathology and clinical symptoms. This knowledge should translate to improved targets for disease-modifying therapies and a better understanding of how to measure the contribution of other genetic and environmental risk factors as well as the efficacy of disease-modifying treatments in physiologically relevant in vivo model systems. The long-term impact will hopefully include facilitating novel clinical treatments that could slow or prevent dementia in PD, which would greatly enhance health for patients and caregivers, reduce disability and caregiver cost, and possibly lengthen life.

We have developed a method for measuring disruption of FC in mouse cortex following induction of spreading of aSyn pathology using an aSyn PFF injection model. This technique should facilitate multiple additional projects in the future to examine the role of specific risk factor genes and the effect of disease-modifying therapies for PD, PDD, and DLB.

We changed our Standard Operating Procedure for preparation and purification of aSyn recombinant protein based on knowledge gained during this project and anticipate that this will result in more reliable assessment of pathology specifically induced by aSyn aggregation rather than non-specific inflammation due to endotoxin contamination.

   - **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.

   - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**.

     Nothing to report.

   - **Significant changes in use or care of human subjects**

     Not applicable – no human subjects.

   - **Significant changes in use or care of vertebrate animals.**

     Nothing to report.

   - **Significant changes in use of biohazards and/or select agents**

     Nothing to report.

6. **PRODUCTS:**
   - **Publications, conference papers, and presentations**.
     - **Journal publications.**

       Nothing to report.

- **Books or other non-periodical, one-time publications.**

  Nothing to report.

- **Other publications, conference papers, and presentations.**

  Nothing to report.

- **Website(s) or other Internet site(s)**.

  Nothing to report.

- **Technologies or techniques**.

  Nothing to report.

- **Inventions, patent applications, and/or licenses**.

  Nothing to report.

- **Other Products**

  Nothing to report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
   - **What individuals have worked on the project?**

Name:	Albert Augustus Davis
Project Role:	Principle Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0003-2042-8445
Nearest person month worked:	0.6
Contribution to Project:	Dr. Davis planned and performed experiments including protein purification and animal surgeries and analyzed data.
Funding Support:	N/A
Name:	Adam Quentin Bauer
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-8364-3209
Nearest person month worked:	0.73
Contribution to Project:	Dr. Bauer planned fcOIS experiments and analyzed data.
Funding Support:	N/A
Name:	Annie Bice

Project Role:	Research Lab Supervisor
Researcher Identifier (e.g. ORCID ID):	not available
Nearest person month worked:	0.43
Contribution to Project:	Ms. Bice performed fcOIS experiments and analyzed data.
Funding Support:	N/A

Name:	Jessica N. Patterson
Project Role:	Technician/Staff Scientist
Researcher Identifier (e.g. ORCID ID):	0000-0003-2560-6546
Nearest person month worked:	2.39
Contribution to Project:	Ms. Patterson supervised mouse colonies, performed protein purification, and analyzed histology data.
Funding Support:	N/A

Name:	Pamela Lynn Cole
Project Role:	Research Technician I
Researcher Identifier (e.g. ORCID ID):	Not Available
Nearest person month worked:	1.18
Contribution to Project:	Ms. Cole assisted with protein purification, managed mouse colonies, and performed histology.
Funding Support:	N/A

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

  Dr. Davis: RF1AG083753 started 9/1/2023

- **What other organizations were involved as partners?**

  Nothing to report.

8. **SPECIAL REPORTING REQUIREMENTS**

   Not applicable

9. APPENDICES

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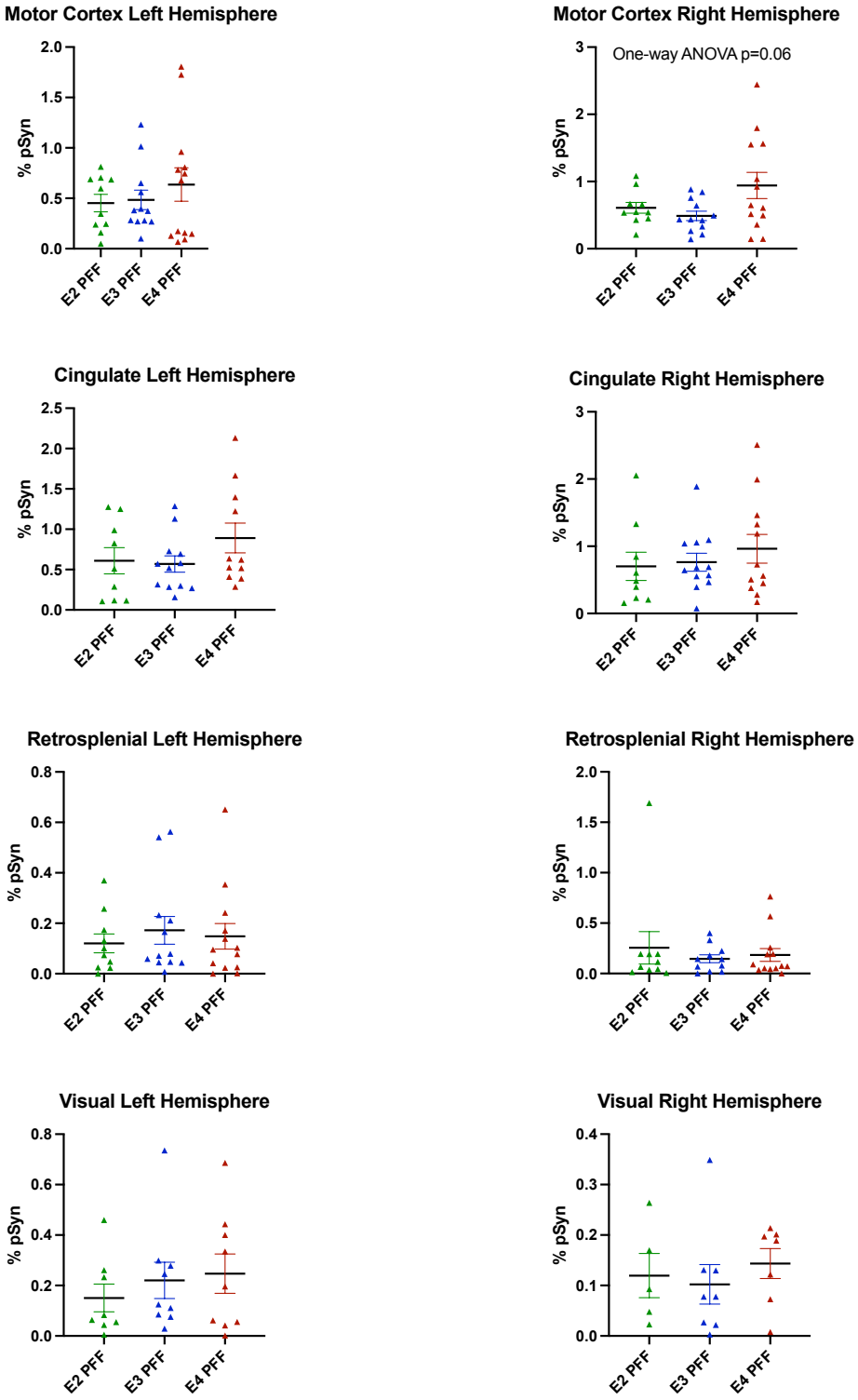

Figure 1. aSyn pathology in cortical brain regions of APOE-knockin mice injected with aSyn PFFs at 3-4 months and aged for 6 months. aSyn pathology was visualized by immunohistochemistry using an antibody that recognizes hyperphosphorylated aSyn aggregates (pSyn) and pathology was quantified by measuring the percent area covered in the indicated brain regions. No groups showed statistically significant differences in this experiment.

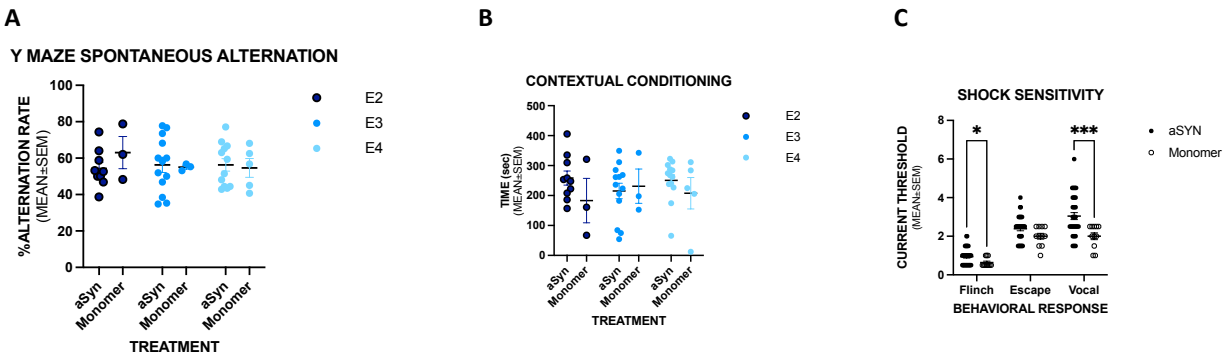

Figure 2. Cognitive behavior in APOE-knockin mice injected with aSyn monomer or PFFs at 3-4 months and aged for 6 months. A) Y maze spontaneous alteration shown as percent of alternation rate. **B)** Freezing time for contextual fear conditioning. **C)** Shock sensitivity for conditioned fear testing expressed as current threshold. * $p<0.05$ and *** $p<0.001$ by two-way ANOVA with Sidak's multiple comparison test.

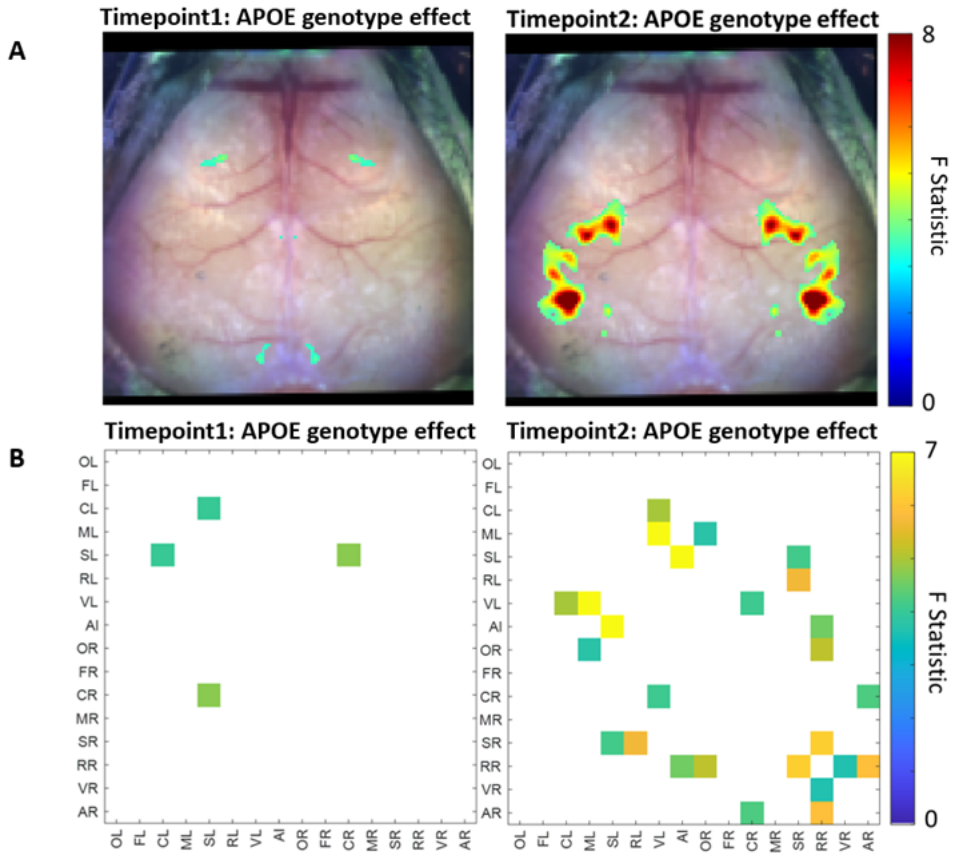

Figure 3. aSyn PFF-induced changes in functional brain organization over time, and effect of APOE genotype. Resting state functional connectivity (RSFC) mapping was applied to 3 groups of mice expressing different isoforms of the APOE gene, injected with aSyn PFFs at 3-4 months of age, and imaged at 2 different time points (3 vs 6 months after injection for timepoint 1 vs 2, respectively). N= 15 mice in each group. **A)** Maps of bilateral FC or **B)** seed-based regional FC at each time point (columns) were subject to one-way ANOVA to determine effect of APOE gene on alpha-synuclein pathology. Significant effects are plotted as F-statistics. All colored clusters/pixels indicate a $p<0.05$. Seed-based FC calculated in the left (L) and right (R) hemispheres for cortical regions corresponding to Olfactory (O), Frontal (F), Motor (M), Somatosensory (S), Retrosplenial (R), Visual (V) and Auditory (A) cortex.

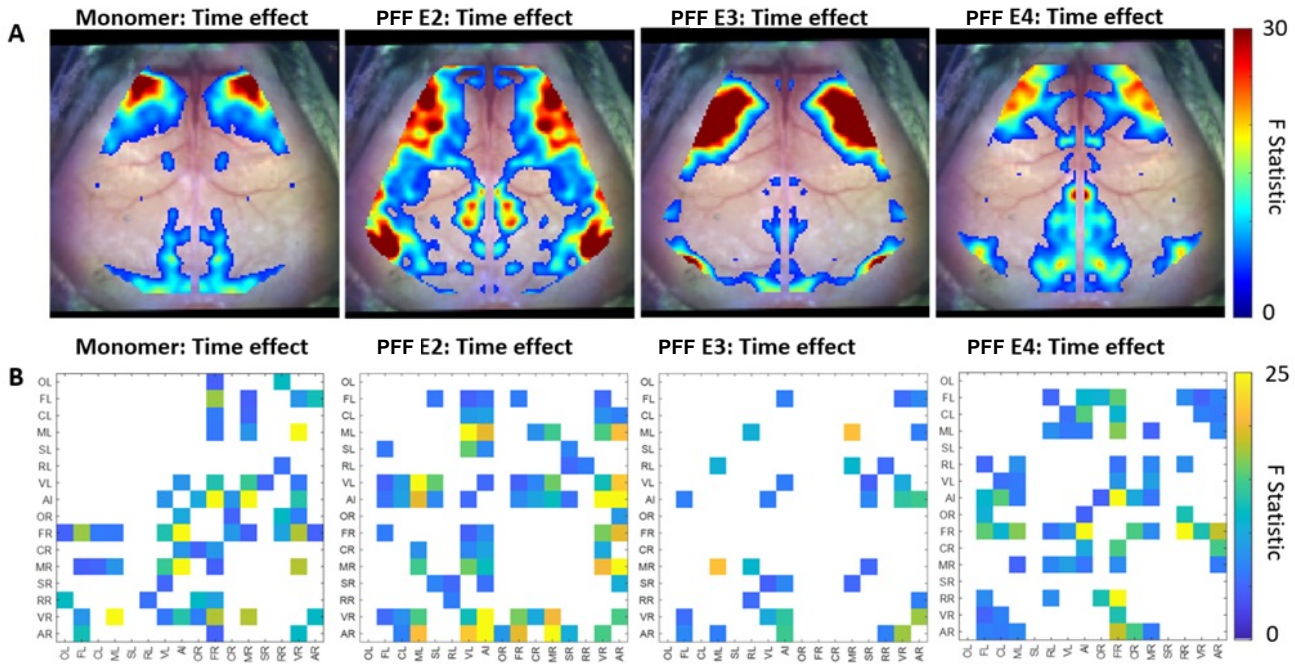

Figure 4. Changes in functional brain organization over time in APOE knockin mice injected with aSyn PFFs. Resting state functional connectivity (RSFC) mapping was applied to 4 groups of mice expressing different isoforms of the APOE gene, injected with either aSyn monomer or PFFs at 3-4 monnths of age, and imaged at 2 different time points (3 vs 6 months after injection for timepoint 1 vs 2, respectively). N= 15 mice in each group . **A)** Groupwise maps of bilateral FC or **B)** seed-based regional FC for each group (columns) were subject to one-way ANOVA to determine effect of time on aSyn pathology. Significant effects are plotted as F-statistics. All colored clusters/pixels indicate a $p<0.05$. Seed-based FC calculated in the left (L) and right (R) hemispheres for cortical regions corresponding to Olfactory (O), Frontal (F), Motor (M), Somatosensory (S), Retrosplenial (R), Visual (V) and Auditory (A) cortex.

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