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TITLE: USP13-Mediated Dopaminergic Neurodegeneration

PRINCIPAL INVESTIGATOR: Hanseok Ko

CONTRACTING ORGANIZATION: Johns Hopkins University, Baltimore, MD

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Fort Detrick, Maryland 21702-5012

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

We hypothesize that USP13 contributes to dopaminergic neurodegeneration and mitochondrial dysfunction in PD through dysregulation of parkin stability and function and that USP13 could be a potential therapeutic target for PD. To address our hypothesis, we propose 1) To determine whether USP13 depletion leads to dopaminergic neurodegeneration, LBs pathology, and motor and mitochondria defects in vivo (Specific Aim 1), 2) To determine the effects of USP13 manipulation (overexpression or depletion) in dopaminergic neurodegeneration, LBs-like pathology, and mitochondrial dysfunction induced by 6-OHDA or alpha-synuclein PFF in human dopaminergic neurons (Specific Aim 2), 3) To determine the effects of USP13 manipulation (overexpression or depletion) in dopaminergic neurodegeneration, LBs-like pathology, and motor and mitochondria defects in the 6-OHDA mouse model or the alpha-synuclein PFF mouse model (Specific Aim 3).

15. SUBJECT TERMS

Ubiquitin, Preformed fibrils (PFF), α-synuclein (α-syn), phosphorylated serine 129 (pSer129), Parkinson's disease (PD), Parkin, USP13, 6-hydroxydopamine (6-OHDA)

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1. INTRODUCTION:

The project is entitled as characterization of the role of USP13 in Parkinson's disease (PD). The major neuropathological hallmarks of PD are progressive with selective loss of DA neurons, neuronal inclusions, and mitochondrial dysfunction. Mitophagy, a type of selective autophagy promotes mitochondrial turnover and prevents the accumulation of dysfunctional mitochondria to maintain cellular homeostasis. The genes linked to PD, such as PINK1 and Parkin, have been revealed to play a role in the major pathway of mitophagy control. Earlier studies reported dysfunction of PINK1/Parkin-dependent mitophagy resulting in pathogenesis of PD. Previous evidence indicates that not only ubiquitination (addition of ubiquitin on proteins and other molecules) but also deubiquitination (cleaving ubiquitin from proteins and other molecules) plays a role in the regulation of protein stability and signaling in mitophagy. USP13 stabilizes the PTEN protein through deubiquitination of PTEN. Here, we aimed to determine the unexplored role of USP13 in regulating the stability and function of Parkin, a key player in mitophagy. Based on our preliminary data, we hypothesize that USP13 contributes to dopaminergic neurodegeneration in PD by regulating parkin stability required for mitochondria function. We will pursue experimental (Aims 1-3) addressing common research questions. The impact of USP13 deficiency in PD pathogenesis and the effects of USP13 manipulation (overexpression or depletion) on 6-OHDA-induced and pathological alpha-synuclein preformed fibrils (PFF)-induced dopaminergic (DA) neurodegeneration, Lewy bodies (LBs) pathology, motor deficits, and mitochondrial dysfunction due to parkin dysregulation. We hypothesize that USP13 can be potential therapeutic target for PD.

2. KEYWORDS:

Ubiquitin, Pre-formed fibrils (PFF), α-synuclein (α-syn), phosphorylated serine 129 (pSer129), Parkinson's disease (PD), Parkin, USP13, 6-hydroxydopamine (6-OHDA)

3. ACCOMPLISHMENTS:

Major goals of the project (from approved SOW):

Specific Aim 1: Can USP13 deficiency drive dopaminergic neurodegeneration?
Specific Aim 2: To determine the effects of USP13 manipulation in dopaminergic neurodegeneration, LB pathology, and mitochondria defects induced by 6-OHDA or alpha-synuclein PFF in human dopaminergic neurons.
Specific Aim 3: To determine the effects of USP13 manipulation in dopaminergic neurodegeneration, LB pathology, motor and mitochondria defects in 6-OHDA intoxication mouse model or alpha-synuclein PFF mouse model.

What was accomplished under these goals?

ACCOMPLISHMENTS

Specific Aim 1: Can USP13 deficiency drive dopaminergic neurodegeneration?

Subtask 1, 2, 3 , and 4 – Under Process and expect to finish by March 2023
Within this age-dependent effect on USP13 k/o mice

We are taking the 3 different cohorts:
1. 6 months cohort: aged matched WT mice and USP13 mice: completed neurobehavioral analysis: Pole Test, Rota rod test, Grip Strength test, Biochemical analysis: Under process

2. 12 months cohort: aged matched WT mice and USP13 mice: completed neurobehavioral analysis: Pole Test, Rota rod test, Grip Strength test, Biochemical analysis: Under process

3. 18 months cohort: aged matched WT mice and USP13 mice: To complete in Feb 2023 for neurobehavioral analysis: Pole Test, Rota rod test, Grip Strength test, Biochemical analysis: HPLC, mitochondrial assays, histopathological studies and analysis

Specific Aim 2: To determine the effects of USP13 manipulation in dopaminergic neurodegeneration, LB pathology, and mitochondria defects induced by 6-OHDA or alpha-synuclein PFF in human dopaminergic neurons.

Subtask 1: **Collaboratively working with Dr. Lee and Dr. Kim**

• Maintain human H9 embryonic stem cells (hECSc) (WA09, WiCell Research Institute, Inc.)

• Generation of USP13 KO hESCs lines by Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR-associated protein 9 (Crispr/Cas9) technology

• Approved JHU electronic institute stem cell research oversight (eISCRO) number: ISCRO00000064

Subtask 2: **Collaboratively working with Dr. Lee and Dr. Kim**

• Derivation of midbrain dopaminergic (DA) neurons from human USP13 KO hESCs lines and control lines

• Purify Lenti-USP13 virus

• Treat 6-OHDA or alpha-synuclein PFF into human DA neurons with USP13 depletion

• Co-treat Lenti-USP13 virus/6-OHDA or Lenti-USP13 virus/alpha-synuclein PFF into human DA neurons

Subtask 3: **Collaboratively working with Dr. Lee and Dr. Kim**

• Conduct IHC, WB analysis, TEM analysis to assess pSer129 α-synuclein immunoreactivity, loss of dopaminergic neurons, and mitochondrial defects

• Conduct cell death assay and Seahorse analysis

• Assess the protein levels of USP13, parkin and its substrates

Subtask 4: **Collaboratively working with Dr. Lee and Dr. Kim**

• Assess the effects of USP13 manipulation on neuron-to neuron transmission of pathologic alpha-synuclein using microfluidic chamber

Subtask 5: **Collaboratively working with Dr. Lee and Dr. Kim**

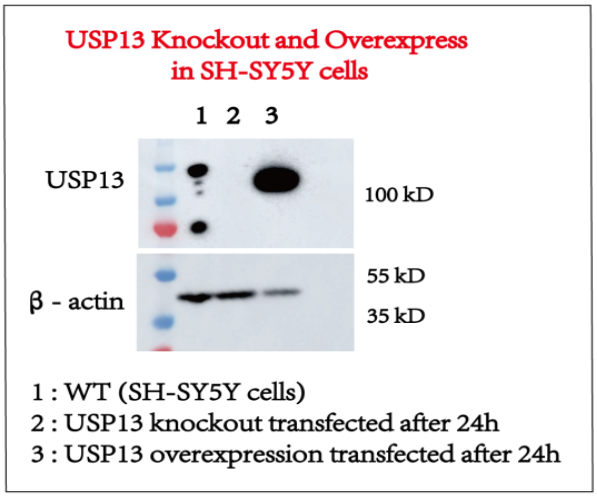
Data Analysis

Milestone(s) Achieved: **Collaboratively working with Dr. Lee and Dr. Kim**

• Determined the effects of USP13 manipulation (depletion or overexpression) in Lewy bodies (LBs)-like pathology, synaptic dysfunction, neurotoxicity, and mitochondria defects as well as parkin protein levels and its substrates accumulation induced by 6-OHDA or alpha-synuclein PFF in human DA neurons.

Obtained Results for Specific Aim 2

A



B

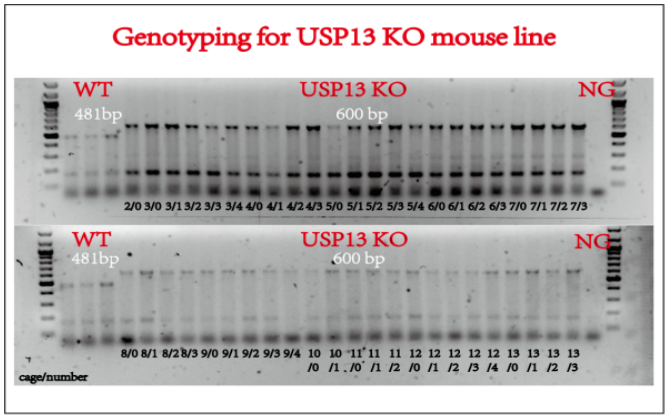


Figure 1 (A). SH-SY5Y cells were transfected with Flag-USP13, USP13 gRNA 1 and 2 to generate USP13 overexpressed and knockout cells. Western blot was used to determine the USP13 expression in WT, USP13 overexpression, USP13 KO SH-SY5Y cells. β-actin was mesured as a reference protein. (B). The genotyping for USP13 KO mouse was carried out by PCR. The WT mice have a bind of 481bp size and USP 13 KO mice have a bind of 600 bp. NG: negative control.

C

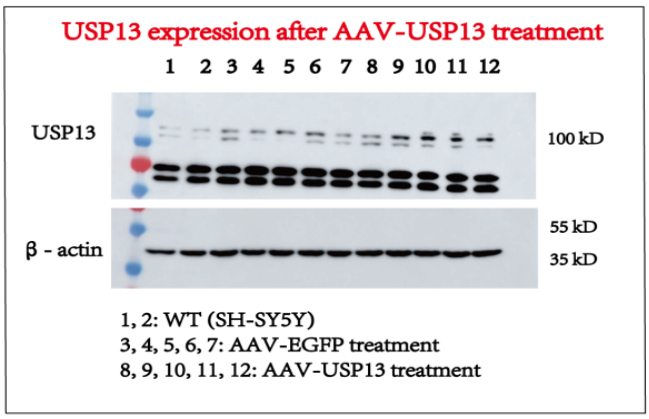


Figure 1 (C). AAV-EGFP and AAV-USP13 were injected in the SH-SY5Y cells with different viural titers. After AAV-USP13 injection, USP13 expression was increased. The viural titer of 2*10^10 VG showed significantly increased in the SH-SY5Y cells.

D

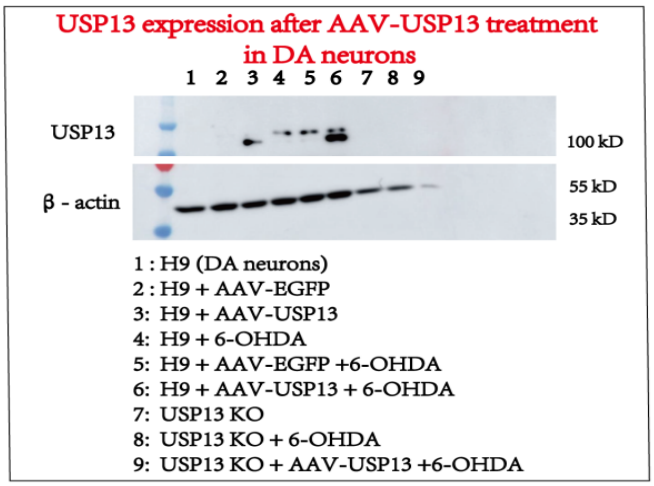


Figure 1 (D). AAV-EGFP and AAV-USP13 were injected in the hDA neurons with viural titer of 2*10^10 VG. Injected with AAV-USP13 increased the USP13 expression, but it showed no different in USP13 KO group.

Figure 1

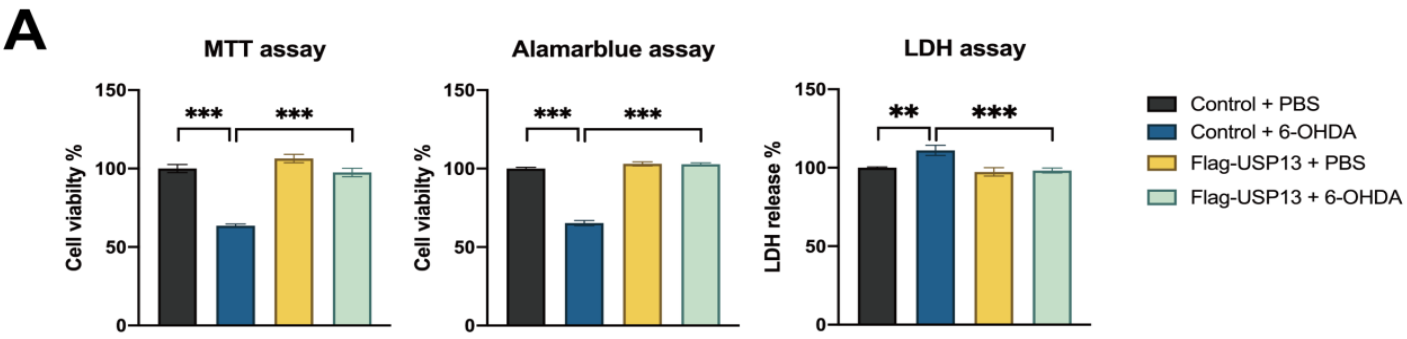


Figure 2 (A). WT and USP13 overexpressed SH-SY5Y cells were treated with 6-OHDA with an concerntration of 200μM incubated for 20h. Cell viability was meausred by MTT, Alamarblue and LDH assay. WT treated with 6-OHDA showed reduced cell viablity compared with control group. USP13 overexpressed SH-SY5Y cells displayed a increased cell viablity compared with WT treated with 6-OHDA group. t-test was performed.*p<0.05, **p<0.01,***p<0.001.

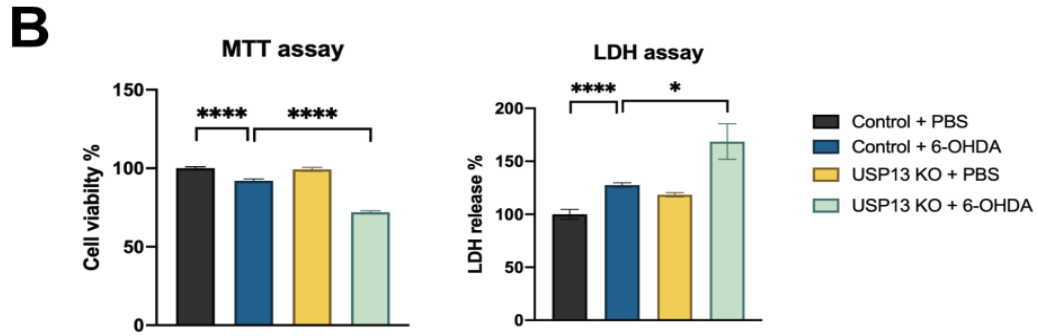


Figure 2 (B). WT and USP13 KO SH-SY5Y cells were treated with 6-OHDA with an concerntration of 200μM incubated for 20h. Cell viability was meausred by MTT and LDH assay. USP13 KO SH-SY5Y cell treated with 6-OHDA showed a decreased cell viablity compared with WT treated with 6-OHDA group. t-test was performed.*p<0.05, **p<0.01,***p<0.001.

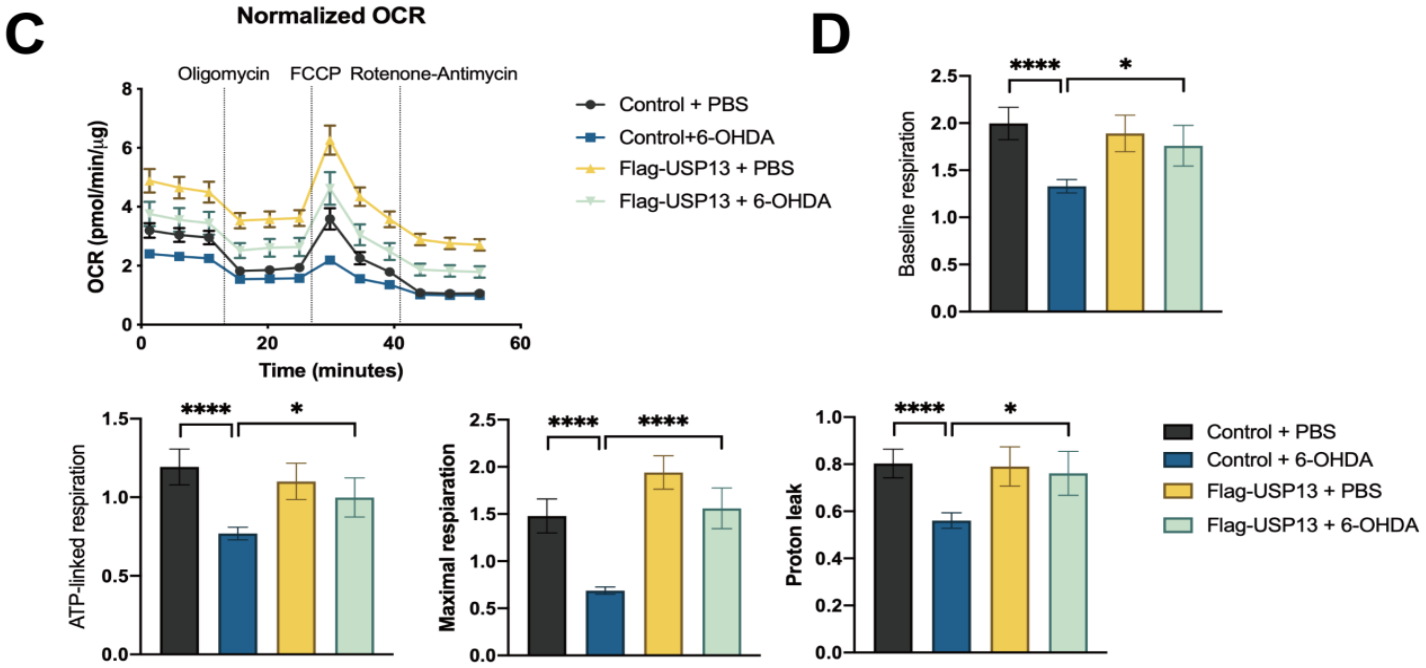


Figure 2 (C). Seahorse assay was carried out in WT and USP13 overexpressed SH-SY5Y cells. These cells were also treated with 6-OHDA with an concerntration of 200μM incubated for 20h. WT treated with 6-OHDA showed reduced basal、ATP-linked、maximal respiration and proton leak compared with control group. USP13 overexpressed SH-SY5Y cells displayed increased OCR compared with WT treated with 6-OHDA group. t-test was performed.*p<0.05, **p<0.01,***p<0.001.

Figure 2

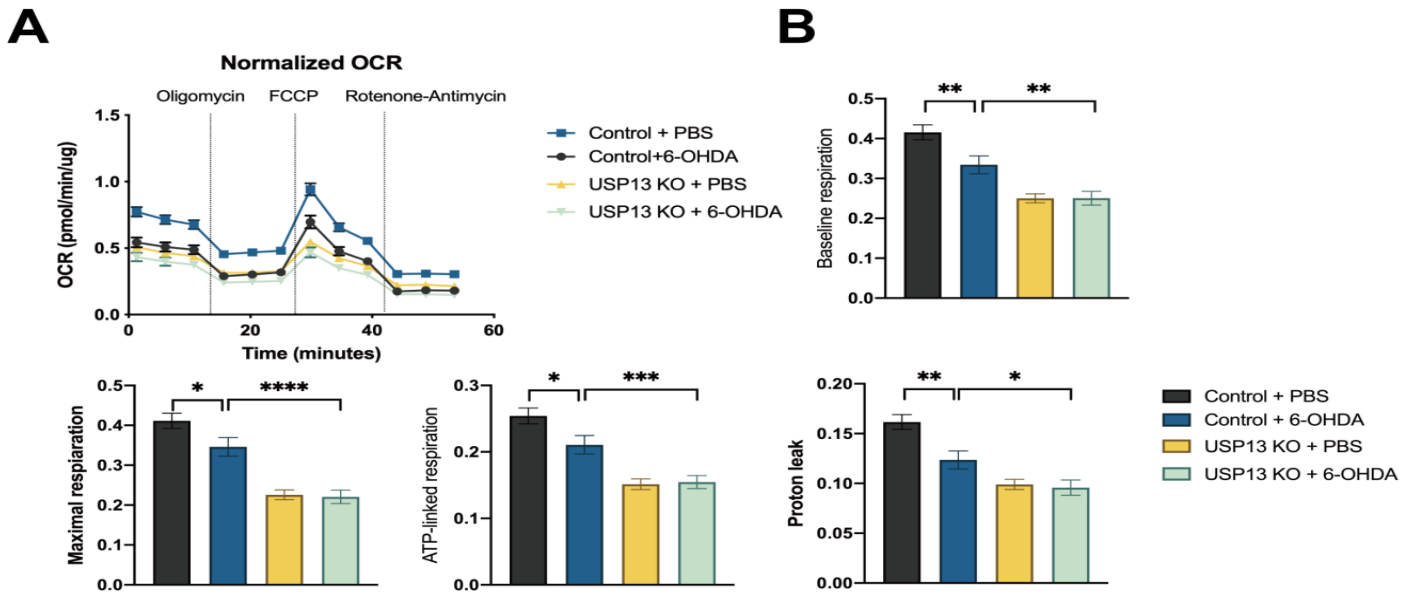


Figure 3 (A). Seahorse assay was carried out in WT and USP13 KO SH-SY5Y cells. The cells were treated with 6-OHDA with an concerntration of 200μM incubated for 20h. (B). WT treated with 6-OHDA showed reduced basal、ATP-linked、maximal respiration and proton leak compared with control group. USP13 KO SH-SY5Y cells displayed decreased OCR compared with WT treated with 6-OHDA group. t-test was performed.*p<0.05, **p<0.01,***p<0.001.

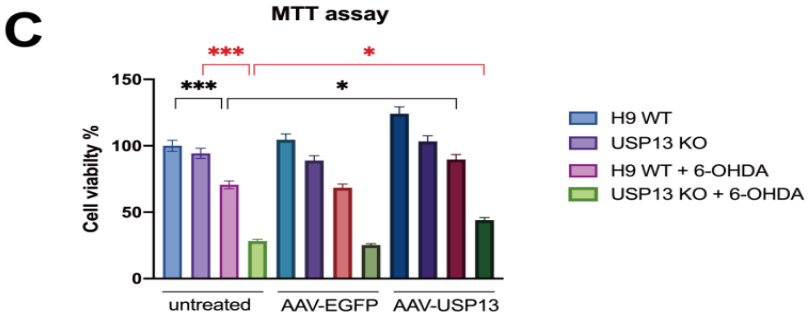


Figure 3 (C). H9 and USP13 KO human DA neurons were treated with 6-OHDA with an concerntration of 200μM incubated for 20h. Cell viability was meausred by MTT assay. USP13 KO human neurons treated with 6-OHDA showed a decreased cell viablity compared with H9 treated with 6-OHDA group, but increase treated with AAV-USP13. t-test was performed.*p<0.05, **p<0.01,***p<0.001.

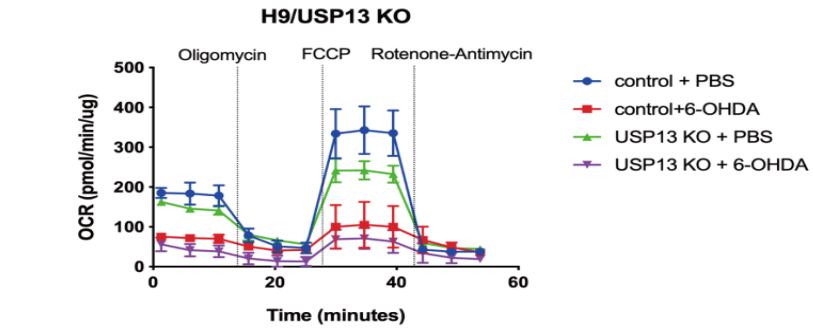


Figure 3 (D). Seahorse assay was carried out in H9 and USP13 KO human DA neurons. The cells were also treated with 6-OHDA with an concerntration of 200μM incubated for 20h. H9 treated with 6-OHDA showed reduced OCR compared with control group. USP13 KO human DA treated with 6-OHDA displayed reduced OCR compared with H9 treated with 6-OHDA group.

Figure 3

Specific Aim 3: To determine the effects of USP13 manipulation in dopaminergic neurodegeneration, LB pathology, motor and mitochondria defects in 6-OHDA intoxication mouse model or alpha-synuclein PFF mouse model.

Subtask 1

• Establish the animal protocols and get them ratified by DoD: **Completed**

Subtask 2

• Collect cohorts of USP13 (C57BL/6) KO mice: **Done**

• Generate alpha-synuclein PFF and purify adeno-associated virus (AAV)-USP13 virus: **Completed**

Subtask 3

• Inject 6-OHDA or alpha-synuclein PFF into the striatum of USP13 KO mice 6-OHDA dose in AAV:
Partially done but further need to be done

• Co-inject AAV-USP13 virus and 6-OHDA or AAV-USP13 virus and PFF into the striatum of wild type (WT) mice: **Partially done but further need to be done**

Subtask 4

• Conduct behavioral tests to assess motor deficits:
To repeat with new experiments in different cohorts

• Sacrifice animals at defined time points and collect brain tissues:

To repeat with new experiments in different cohorts

• Conduct IHC, WB analysis, TEM analysis to assess pSer129 α-synuclein immunoreactivity, loss of dopaminergic neurons, and mitochondrial defects:
Will be done after initial experiment setup completed

• Assess the protein levels of USP13, parkin and its substrates:
Will be done after initial experiment setup completed

• We will use at least n=20 mice for behavior tests, and n=6~8 for neuropathological and neurobiochemical assessment including IHC, WB, and TEM. Both male and female mice will be used for these assays:
Will be done after initial experiment setup completed

• JHU animal protocol number: MO20M52:
Obtained

Obtained Results for Specific Aim 3

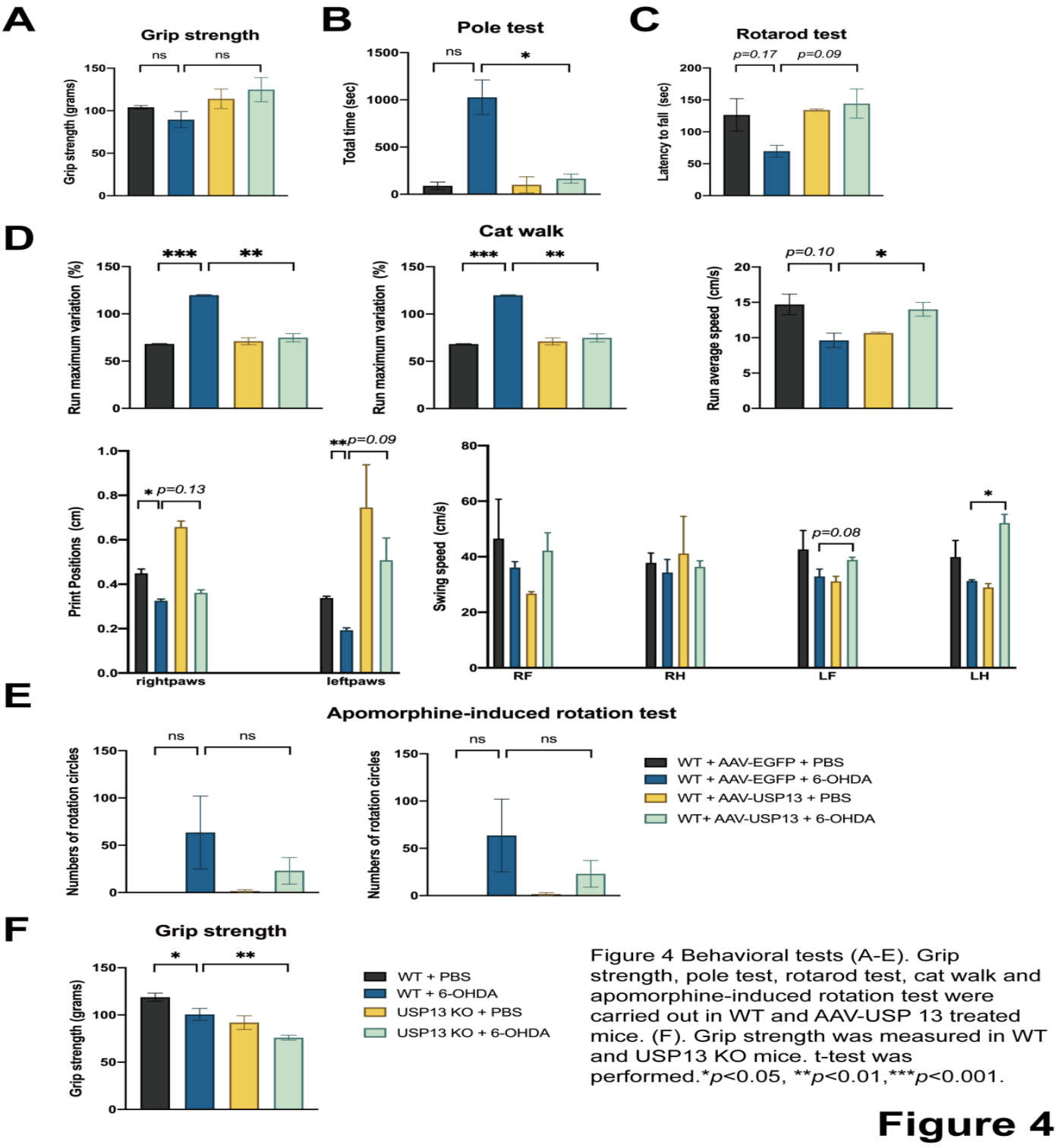


Figure 4 Behavioral tests (A-E). Grip strength, pole test, rotarod test, cat walk and apomorphine-induced rotation test were carried out in WT and AAV-USP 13 treated mice. (F). Grip strength was measured in WT and USP13 KO mice. t-test was performed.*p<0.05, **p<0.01,***p<0.001.

Figure 4

We are working in 2 different cohorts where 6-OHDA and alpha-synuclein PFF model.

6-OHDA intoxication: In process

Alpha-synuclein PFF model: injected PFF (5µg) in striatal region of mice: Aug 2022: long wait for 6 months (till Feb 2023) for neurobehavioral parameters estimation followed by neurochemical assessments such as neurotransmitters via HPLC, mitochondrial assays, Western blot, PCR, histopathological studies including cresyl violet, IHC, IF.

4 Groups (n=48, 12 weeks old, both males and females, C57bl/6 WT mice and C57bl/6 background knockout mice)

Group1: WT+PBS

Group2: WT+PFF

Group3: USP13k/o + PBS

Group4: USP13k/o + PFF

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

Nothing to Report

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?

Characterize of USP13 knockout mice at 6, 12, and 18 months of age. We will determine the effects of USP13 manipulation in dopaminergic neurodegeneration, LB pathology, and mitochondria defects induced by 6-OHDA or alpha-synuclein PFF in human dopaminergic neurons. We will determine the effects of USP13 manipulation in dopaminergic neurodegeneration, LB pathology, motor and mitochondria defects in 6-OHDA intoxication mouse model or alpha-synuclein PFF mouse model.

4. IMPACT:

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:

Nothing to Report

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

6. PRODUCTS:

Nothing to Report

Publications, conference papers, and presentations

Nothing to Report

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:	Dr. Hanseok Ko
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-0864-5239
Nearest person month worked:	2.4 months per year
Contribution to Project:	Dr. Ko is the principal investigator who is overseeing this project.
Funding Support:	

Name:	Dr. Gabsang Lee
Project Role:	Co-investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-5052-5927
Nearest person month worked:	0.2 months per year
Contribution to Project:	Dr. Lee is the Co-investigator who is working with human DA neurons
Funding Support:	

Name:	Dr. Hyesoo Kim
Project Role:	Co-investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-4314-1327
Nearest person month worked:	1.1 months per year
Contribution to Project:	Dr. Kim is the Co-investigator who is working with human DA neurons
Funding Support:	

Name:	Dr. Jing Wang
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	0000-0003-3419-3990
Nearest person month worked:	6 months per year
Contribution to Project:	Dr. Jing has performed work in the area of injection and behavior assays, cell death, seahorse, Western blot
Funding Support:	

Name:	Dr. A Ra Kho
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	0000-0001-9812-6186
Nearest person month worked:	0.5 months per year
Contribution to Project:	Dr. A Ra Kho has performed work in the area of injection.
Funding Support:	

Name:	Dr. Minsung Kim
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	000-0002-7911-8604
Nearest person month worked:	3 months per year
Contribution to Project:	Dr. Minsung has performed work in the area of DA neurons differentiation and generation of iPSC line with USP13 KO
Funding Support:	

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

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:
Submitted with attachments.

9. APPENDICES
No attachments

Project Title: USP13-Mediated Dopaminergic Neurodegeneration
Log Number: **PD200031** Annual report 1
Award Number: **W81XWH-21-1-0908**

PI: Ko, Hanseok **Org:** Johns Hopkins University **Award Amount:** $732,825/Direct

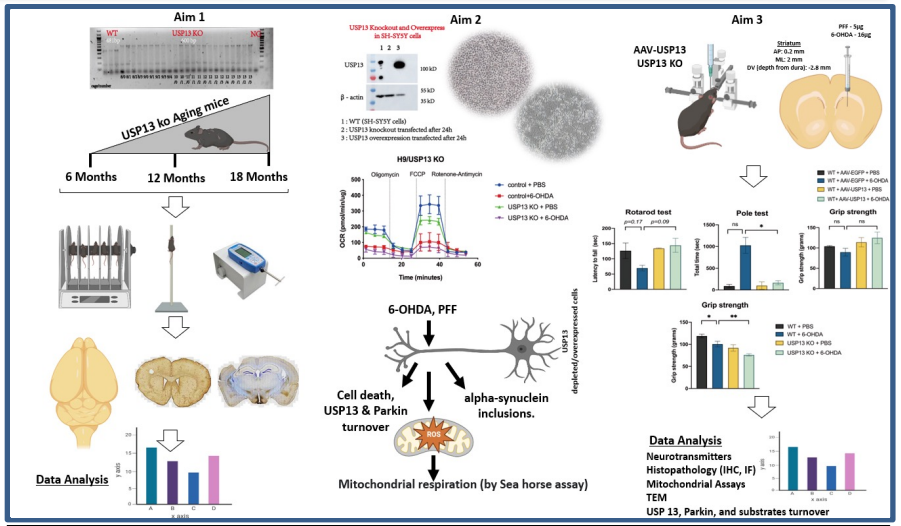
Study Aims

1. Can USP13 deficiency drive dopaminergic neurodegeneration?

2. To determine the effects of USP13 manipulation in dopaminergic neurodegeneration, LB pathology, and mitochondria defects induced by 6-OHDA or alpha-synuclein PFF in human dopaminergic neurons.

3. To determine the effects of USP13 manipulation in dopaminergic neurodegeneration, LB pathology, motor and mitochondria defects in the 6-OHDA intoxication mouse model or alpha-synuclein PFF mouse model.

Approach: This work will broaden our understanding of the previously overlooked but significant pathogenic mechanism of PD caused by neurotoxin exposure, using rodent and human neuron model systems. This research will provide a new insight into the role of USP13 in PD pathogenesis caused by neurotoxin exposure and will, hopefully, lead to a new therapeutic intervention for PD.



Workflow of schematic representation for Aim 1, Aim 2 and Aim3

Timeline and Cost

Activities CY	21-22	22-23	23-24	
Study Prep/Specific Aim 1	$41,425	$61,850	$121,425	
Specific Aim 2 (see goals/milestones)	$121,425	$81,000		
Specific Aim 3 (see goals/milestones)	$81,425	$101,425	$122,850	
Estimated Budget ($732,825)	**$244,275**	**$244,275**	**$244,275**	

Updated: 10/10/2022

Goals/Milestones

CY21-22: 1) Obtain ACUC approval for animal protocol number MO20M52 at JHU; 2) Knockout animals generated and divided into 3 cohorts (6,12, 18 months) for studying USP13 depletion impacts DA neuron defects, mitochondria quality control, parkin downregulation & substrate accumulation: under process

CY21-22: 1) Establishment of USP13 k/o and overexpression system in SHSY-5Y, H9, and human DA neurons and conduction of experiments with 6-OHDA for cell viability, cell death assays, cellular respiration: To continue (with collaborators)…and repeat experiments for reproducibility. 2) Generation of PFFs; 2) PFFs ready to be used; 3) Treatment of cells with PFFs (aim 2): under process 4) data analysis/manuscript prep and submission

CY21-22: 1) Inject mice (co-inject AAV-USP13 and USP13 KO) with 6-OHDA, PFFs (aim 3); 2) Neurobehavioral, biochemical, histological analysis (aim 3); 3) data analysis/manuscript prep and submission

Comments/Challenges/Issues/Concerns

- NA

Budget Expenditure to Date

Projected Expenditure: $244,275 (Direct)
Actual Expenditure: $172,835 (Direct)