AWARD NUMBER: W81XWH-22-1-0584

TITLE: The Role of NADPH Oxidase1 (NOX1) in the Colon in Rotenone-Induced a-Synuclein Pathology Propagation Along the Gut-Brain Axis

PRINCIPAL INVESTIGATOR: Yoon-Seong Kim

CONTRACTING ORGANIZATION: Rutgers, The State University of New Jersey
Newark,NJ

REPORT DATE: July 2023

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| July 2023 | Annual | 01Jul2022-30Jun2023 |

**4. TITLE AND SUBTITLE**

The Role of NADPH Oxidase1 (NOX1) in the Colon in Rotenone-Induced a-Synuclein Pathology Propagation Along the Gut-Brain Axis

**5a. CONTRACT NUMBER**
W81XWH-22-1-0584

**5b. GRANT NUMBER**

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

Dr. Yoon Seong-Kim

E-Mail: yk525@rutgers.edu

**5d. PROJECT NUMBER**
0011761933

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY
33 Knightsbridge Road, 2nd Floor, East Wing,
Piscataway, New Jersey 08854-3925

**8. PERFORMING ORGANIZATION REPORT NUMBER**
Year 1 Annual Report

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**
USAMRAA

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

The proposed study will investigate how Nox1-derived ROS contributes to early-stage α-SYN aggregation in the gut using a cell-to-cell α-SYN propagation assay and gut cell-specific Nox1 deleted mice model. We will also test a potent and selective Nox1 inhibitor, ML171, in our gutbrain PD model. Successful completion of the project will elucidate the molecular mechanisms underpinning how α-SYN aggregation is initiated in the gut and to what extent Nox1 plays a role in this process. Moreover, the proposed study could identify a specific Nox1 inhibitor as a novel therapeutic intervention to prevent gut-driven PD development.

**15. SUBJECT TERMS**
None listed.

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRDC |
| Unclassified | Unclassified | Unclassified | Unclassified | 7 | 19b. TELEPHONE NUMBER *(include area code)* |

## TABLE OF CONTENTS

## 1. Introduction

The proposed study will investigate how Nox1-derived ROS contributes to early-stage α-SYN aggregation in the gut using a cell-to-cell α-SYN propagation assay and gut cell-specific Nox1 deleted mice model. We will also test a potent and selective Nox1 inhibitor, ML171, in our gutbrain PD model. Successful completion of the project will elucidate the molecular mechanisms underpinning how α-SYN aggregation is initiated in the gut and to what extent Nox1 plays a role in this process. Moreover, the proposed study could identify a specific Nox1 inhibitor as a novel therapeutic intervention to prevent gut-driven PD development.

## 2. Keywords

NADPH oxidase1 (NOX1); Oxidative DNA damage; Transcriptional mutagenesis; α-synuclein; Rotenone; Gut-brain axis; 8-oxodG

## 3. Accomplishment

A. Task 1: Investigate the role of Nox1-mediated oxidative stress in α-SYN aggregation in EECs.

In the preliminary study, we showed that the treatment of STC1 cells with rotenone increased ROS generation that was blocked by pan-NOX inhibitor as well as a NOX1 selective inhibitor, ML-171. In this funding period, we tested if exogenous α-SYN preformed fibril (PFF) could activate NOX1-mediated ROS generation in STC1 cells. First, we investigated the kinetics of cellular uptake of Atto647-labeled α-SYN PFF using IncuCyte (Fig.1) and found that STC1 cells readily uptake PFF within 3 hours. Interestingly, PFF (1 μg/ml) induced ROS generation that was partially reduced by ML-171 (50 μM) (Fig. 2).

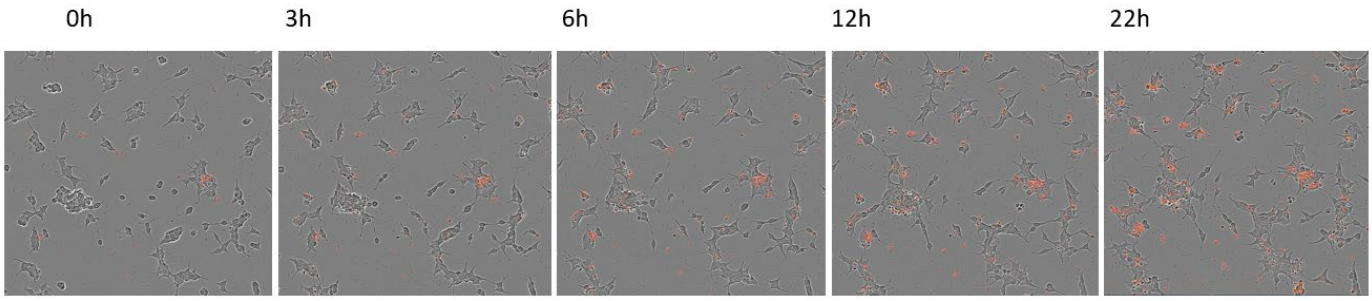


**Fig.1.** STC1 cells were treated by Atto647-labeled human α-SYN PFF (1 μg/ml) and was tranced every 3 hours for its cellular uptake using the IncuCyte.

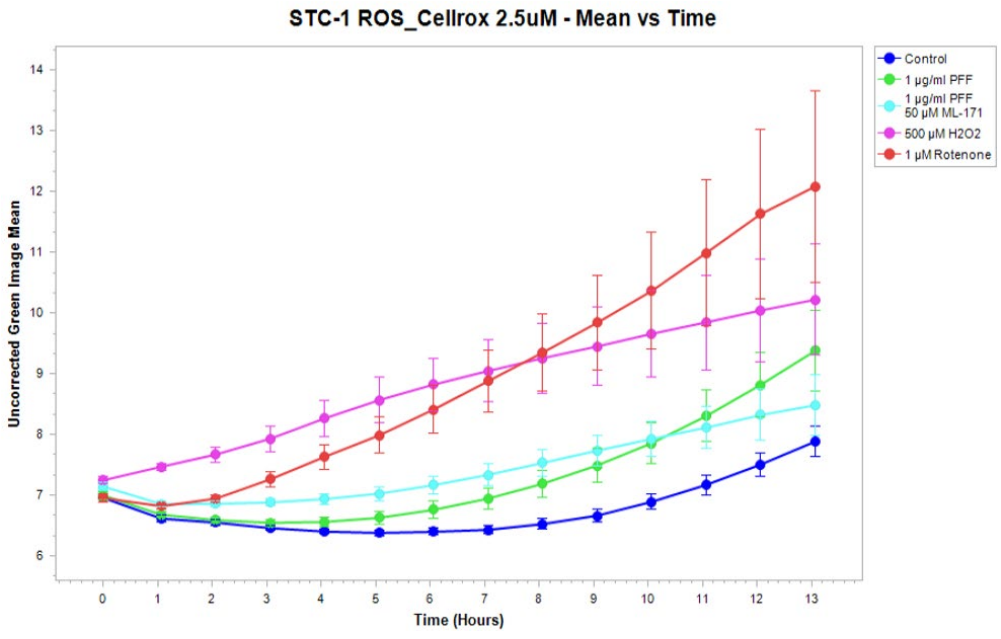


**Fig.2.** α-SYN PFF induces NOX1-mediated ROS generation in STC1 cells. After treatment with various conditions including Rotenone, α-SYN PFF, and α-SYN PFF + ML-171, ROS in STC1 cells were traced overtime using CellRox dye.

This result suggests that α-SYN PFF is uptaken by STC1 cells, causing oxidative stress partially through NOX1 activation. α-SYN PFF-based model could serve as a viable system to test NOX1-mediated α-SYN pathogenesis. Together with the rotenone treatment paradigm that we already established and shown in the preliminary results, this part of the task was completed. (June 16th, 2023).

B. Task2: Investigate propagation of α-SYN from EECs to ENs under Nox1-mediated oxidative stress

We have tried to establish an STC1-IMFEN coculture system. Because their growth conditions are different, we first start to grow IMFEN cells (enteric neuronal cells) and differentiate them followed by the addition of STC1 cells at 37°C. We found that STC1 cells grow well with differentiated IMFEN cells. However, we failed to achieve differentiated IMFEN cells with long neurites, making it challenging to set up a co-culture in a microfluidic system. Now we are testing various coating materials to make them differentiate with long neurite outgrowth.

In the meantime, we have made progress in the NAnoBRET system using STC1 cells. Four different combinations of α-SYN-Nanoluciferase and -Halotag were tested in STC1 cells, demonstrating the HS-NS combination of 1:100 ratio yielded the highest BRET event (Fig.3).

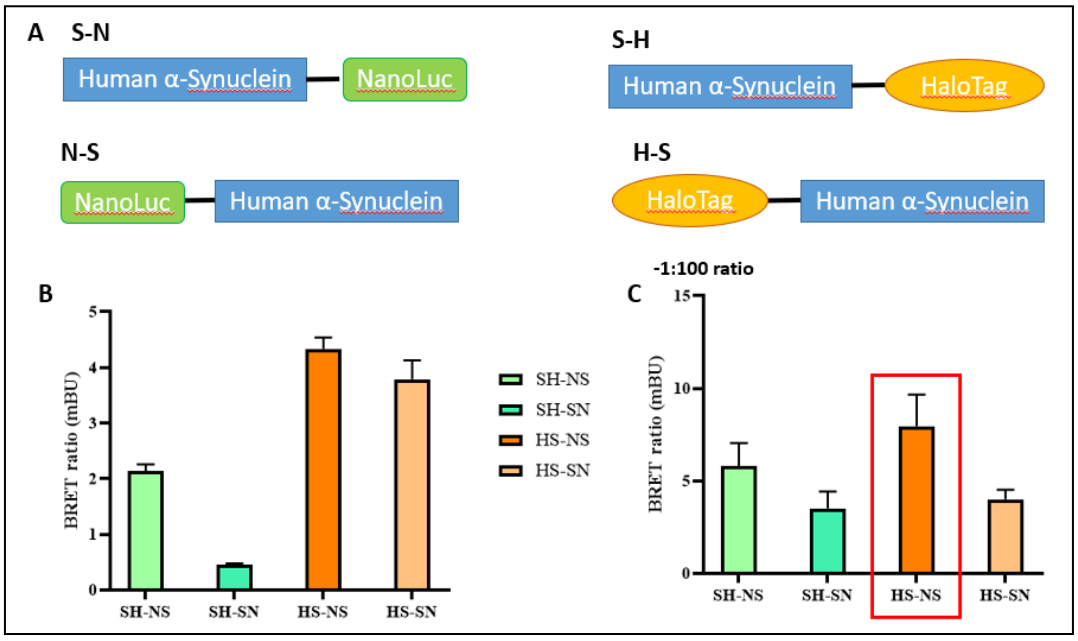


**Fig.3.** NanoBRET system in STC1 cells. α-SYN aggregation monitoring system is established in STC1 cells. A. Four α-SYN plasmids tagged with either Nanoluciferase or Halotag at its N- or C-terminal were constructed.
B. After trascfection to STC1 cells, BRET ratios were measured to determine the highest BRET event, showing a HS-NS as the best one combination. C. The HS-NS with the ratio of 1:100 exhibits the highest BRET event.

This task is currently underway and once the proper co-culture condition of STC1 cells with differentiated IMFEN cells is achieved, we will measure α-SYN transfer from STC1 cells to IMFEN neurons.

C. Task 3: Establish models in Cck-CreERT2:floxed Nox1 mice by intracolonic administration of rotenone or LPS.

As planned, we purchased Ccktm2.1(cre/ERT2)Zjh (Jackson lab, Stock No: 012710), currently expanding the colony to obtain enough size of the cohort of Cre-ERT2:Wt ; $Nox1^{fl}$:$Nox1^{fl}$. To test if tamoxifen-dependent Cre expression in the Cck+ cells, we mated Cre-ERT2 with B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J (Jackson lab, Stock No: 007914). At 6-8 weeks, 75 mg/kg of tamoxifen(TAM) was i.p injected for 3 days, and then tdTomato signal was detected in the colon and brain (Fig.4).

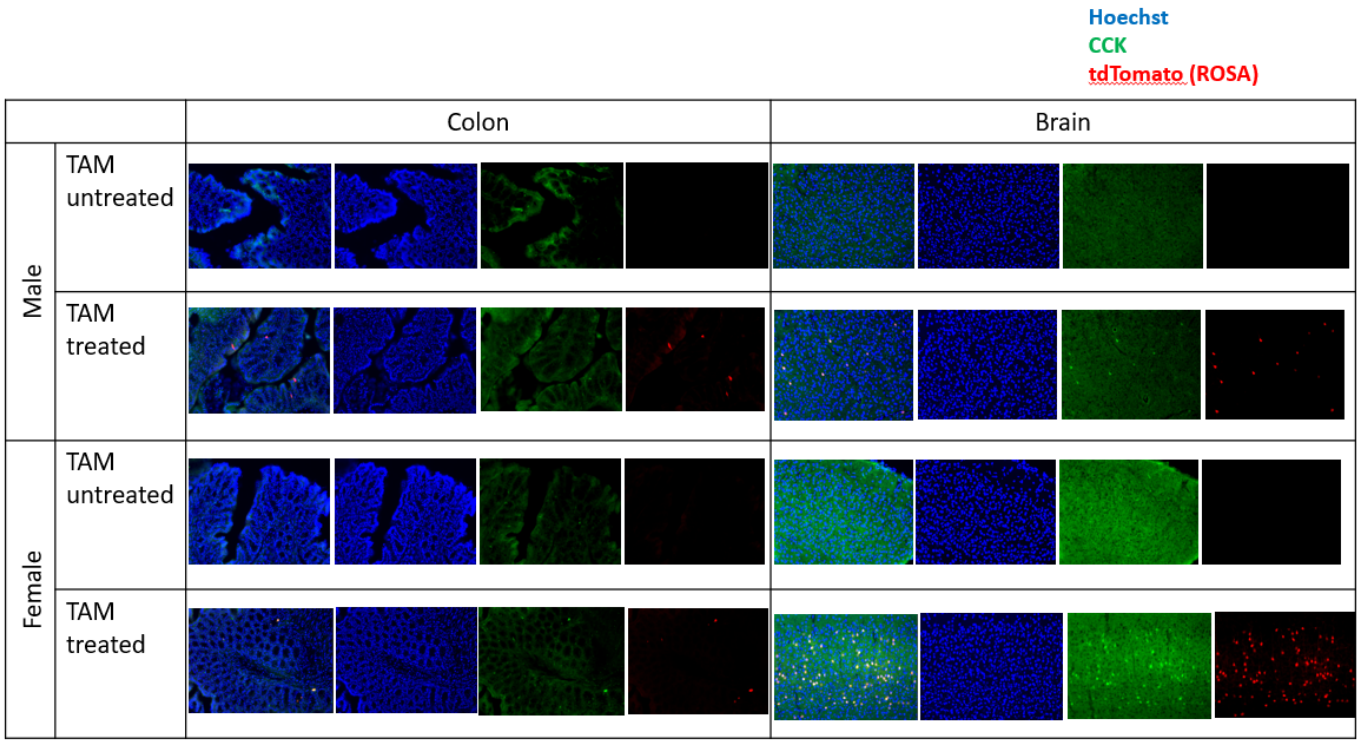


**Fig.4.** Tamoxifen-induced Cck-Cre-dependent loxp recombinase event was confirmed in the brain and colon using B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J crossed with Cck-Cre/ERT2

We are also establishing an additional Gut-Brain PD mouse model. Exogenously injected human α-SYN PFF into the brain parenchyma or duodenal smooth muscle layer can induce the propagation of α-SYN pathology through endogenous α-SYN induction. To develop the Gut-Brain model using α-SYN PFF, we injected mice with Atto633 dye-labeled PFF via colonic enema once per day (9 μg of sonicated PFF in 30μl PBS) for 8 hours, 3 days, 7 days, and I month after 7 days injections. Interestingly, we observed that α-SYN PFF was rapidly taken up in the colon and induced endogenous α-SYN. To our surprise, the Att633 signal (red) was also detected in the dorsal motor nucleus of the vagus (DMV) region in the brain stem as early as 8 hours post-administration (Fig.5).

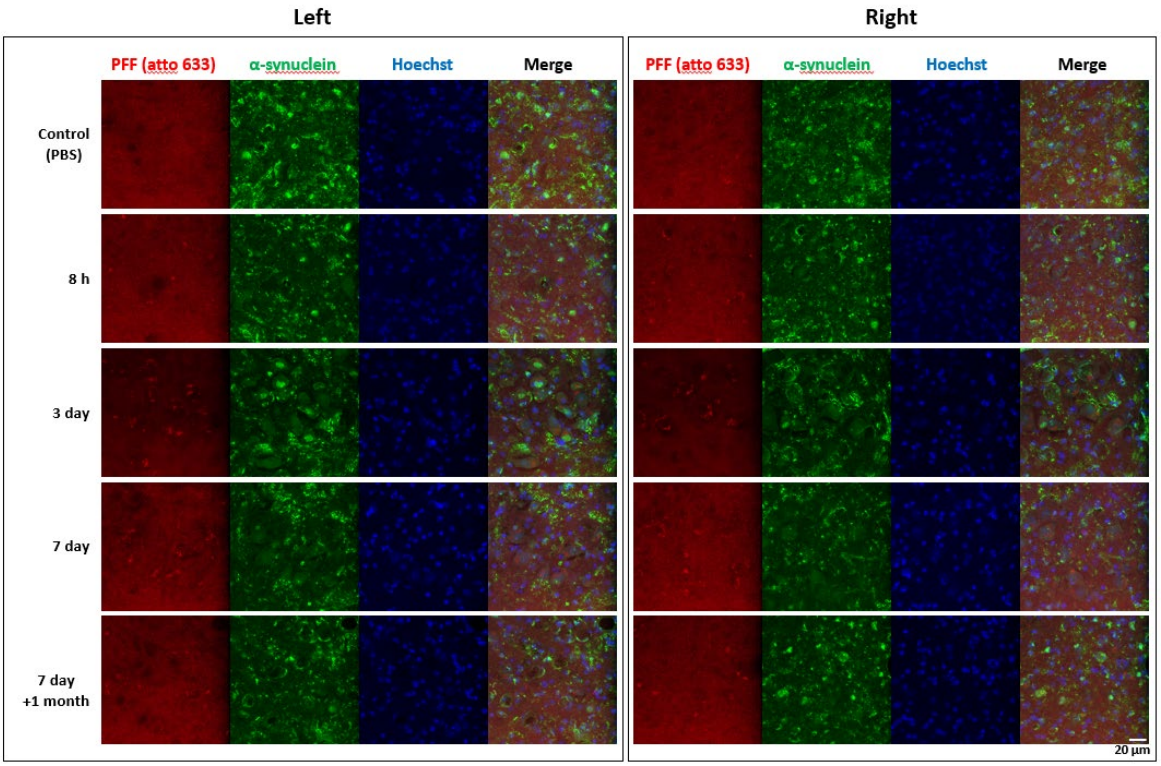


**Fig.5.** The propagation of α-SYN PFF labeled with Atto633 administered by the colonic enema to the DMV.

We are now characterizing pathology in the colon and brain after various durations following 14 days α-SYN PFF colonic enema. This novel model will be used for testing Nox1 inhibitor, ML-171, and conditional Nox1 KO conditions.

### 4. Impact

The colon is the organ where Nox1 expression was well documented. We have established two novel gut-brain PD models that similarly recapitulate gut-to-brain propagation of α-SYN pathology. Using both genetic as well as pharmacological inhibition of Nox1, our ongoing study will test if Nox1 inhibition in the colon will prevent the initiation of α-SYN pathology in the gut and its propagation into the brain.

### 5. Changes/Problems

In the original proposal, we planned to use LPS colonic enema as a second gut-brain PD model for testing Nox1 inhibition. However, we found that colonic administration of LPS did not induce the strong α-SYN pathology in the gut and brain. In addition, it did not induce Nox1-mediated ROS generation and α-SYN induction in STC1 cells. Alternatively, we have started to establish another Gut-Brain model using colonic administration of α-SYN PFF from April. As discussed above in Fig 5, we found that intracolonic administration of α-SYN PFF is rapidly taken up in the colon and induces endogenous α-SYN in the enteric neurons and brain stem. We think this novel model successfully recapitulates gut-brain propagation of α-SYN pathology and Nox1-mediated ROS generation.