Can NoX1 Activity Initiate Parkinson's-Like Pathology in an Enteroendocrine Cell Line?

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EXPLORING A POSSIBLE ROLE FOR NOX1 IN GUT-MEDIATED PARKINSON'S DISEASE PATHOLOGY

BY

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ABSTRACT

Increased attention has been given to the gut lately in a number of conditions, from maintaining health via the use of probiotics to treating Autism Spectrum Disorder. Parkinson's Disease has a history with the gut starting with the Braak hypothesis, in which eminent researcher Heiko Braak observed that the spread of Parkinson's (PD) seemed to occur along either olfactory or enteric neurons prior to reaching the *substantia nigra* in the midbrain, where the classical disease symptoms become evident. Though this finding was largely ignored at the time, the possibility of a gut origin for PD has received interest lately as a growing body of epidemiological and mechanistic research supports a gut-based influence. One key study showed that the presence of a toxin that induces oxidative stress in the intestine is capable of generating protein aggregates that spread to the brain and cause a PD-like pathology. The spread of protein aggregates from gut neurons to the brain has been corroborated in a number of studies. The open question, then, is what type of toxic triggers are capable of causing protein aggregation in the real world, and how do they cause protein aggregation in enteric neurons, which do not directly contact the intestinal lumen? We propose here that the enzyme Nox1 contributes to oxidative stress in the gut and eventually to the protein aggregation that can lead to PD via the generation of endogenous reactive oxygen species. Nox1 functions to generate superoxide radicals and is highly expressed in the colon. Knockdown of *NOX1* in the brain has been shown to have a protective effect in a PD mouse model. To bridge a trigger in the intestinal lumen to protein aggregation in enteric neurons, we investigate a class of cells that contact both the intestinal lumen and enteric neurons, known as enteroendocrine cells. Finally, we conduct a small study to explore a possible toxic trigger, high fat diet.

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LIST OF ACRONYMS/ABBREVIATIONS

PD – Parkinson's Disease

HFD – High Fat Diet

OS – Oxidative Stress

ROS – Reactive Oxygen Species

Nox1 – NADPH Oxidase 1

SN – *Substantia Nigra*

EEC – Enteroendocrine Cell

PQ - Paraquat

MPTP – 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine

6-OHDA – 6-Hydroxydopamine

IHC – Immunohistochemistry

PCR – Polymerase Chain Reaction

2ab – Secondary Antibody

LPS – Lipopolysaccharide

TH – Tyrosine Hydroxylase

a-SYN – Alpha Synuclein

INTRODUCTION

Parkinson's Disease (PD) is the second most common neurodegenerative disease, affecting about 1% of the population over 60 and then again to 4% for those over 80 (1). The disease is typically characterized by the progressive loss of dopaminergic neurons in a region of the brain known as the *substantia nigra* or "black substance" (2). Post-mortem analysis of PD brains reveals the only physical hallmark of the disease, insoluble inclusions known as Lewy Bodies present throughout affected brain regions (2). Patients with PD typically present with two of four cardinal symptoms of PD, which include resting tremor, bradykinesia, rigidity, and postural instability (2). The diagnosis of PD is made based on these symptoms, patient history, and the United Parkinson's Disease Rating Scale (2).

The etiology of PD is obscure. Overall, about 80-90% of PD cases are considered idiopathic, meaning that the cause is unknown, and the other approximately 10-20% of cases are linked to genetics (2). In certain cases, the environment seems to play the dominant role, and likely accounts for some of those "idiopathic" cases. For example, exposure to pesticides like rotenone and paraquat increase PD risk by about 70%, and these same pesticides are used in the lab to generate *in vivo* and *in vitro* models of PD since they can recapitulate many of the symptoms and molecular aspects of the disease (3). On the other hand, genetics can also play a major role. Autosomal dominant mutations in *LRRK2* and autosomal recessive mutations in *DJ-1* often result in early-onset PD (2). Despite the many gene loci identified with PD risk and the cases where the environment is known to play a role, the explicit cause of PD in the majority of the cases is unknown.

Briefly, there is one protein that deserves special attention with respect to PD pathology: alpha synuclein (4). Alpha synuclein plays a central role in PD and is extensively studied. The interest for this protein is derived from the fact that it makes up a major component of Lewy Bodies, and causes early onset PD in a dose-dependent manner (4). That is, duplications in *SNCA*, the gene for alpha-synuclein, cause early onset PD, and triplications of *SNCA* result in even *earlier onset* PD (4). At the molecular level, alpha synuclein is thought to contribute to PD when it takes on a beta-sheet conformation instead of its usual alpha helix, which allows it to aggregate into toxic oligomers, while also inducing other alpha synuclein species to fold into beta sheets as well (4). For this reason, alpha synuclein aggregation is referred to as "prion-like" (4). In fact, not only does alpha synuclein spread in a prion-like fashion inside the cell, but it can propagate between cells too. One relatively recent study found that, when PD brains were grafted with stem cells, these new stem cells also developed alpha synuclein aggregates within a matter of years (5).

Recently, mounting evidence has suggested a role for the gut in PD pathogenesis. The foundation for this hypothesis comes from several observations. First, non-motor symptoms of PD, namely constipation, pre-date the motor symptoms by several years (6). As a result, constipation is thought to indicate an early stage of PD pathology, presumably one in which enteric nerves have been damaged by the spread of the disease. Second, alpha synuclein aggregates have been shown to be able to propagate up the vagus nerve to the brain (6). Next, a large study found that patients that had undergone vagotomy were 50% less likely than controls to develop PD (6). Interestingly, the effect was noted only for truncal vagotomy (entire vagus nerve severed) and not for superselective vagotomy (only specific branches of the nerve severed)

(6). This research suggests what is known as “the gut hypothesis for PD”, which posits that a toxic insult in the intestines causes a change in enteric neurons that initiates alpha synuclein aggregation, the aggregates then spread in a prion-like fashion up the vagus nerve into the brain, and finally the alpha synuclein aggregates reach the *substantia nigra* (SN) and induce the motor symptoms that define the disease (6).

In a parallel vein, it is also vital to consider the role that oxidative stress (OS) plays in PD pathology. OS plays a central role in the pathology of many neurodegenerative diseases, including PD (17). In PD specifically, the importance of OS can be illustrated by its core involvement in the major toxin models used to replicate PD pathology including Paraquat, 6-OHDA, MPTP, and Rotenone (18,19). In addition, genetic forms of PD often have some relatedness to OS (18,19). For example, *LRRK2* mutations are related to mitochondrial dysfunction, and *DJ-1* is a quencher of reactive oxygen species (ROS) that is protective against 6-OHDA (18,19). The mechanisms whereby OS would lead to dopaminergic cell death include damage to the ubiquitin-proteasome system, lipid peroxidation, DNA damage, protein damage, alpha synuclein aggregation, and mitochondrial dysfunction (20). There is then the problem of trying to discern whether OS causes or contributes to PD pathogenesis. The issue is that many of the toxic effects that can be mediated by OS can also cause OS themselves (20). Alpha synuclein aggregation, for example, results in the production of ROS that contributes to OS, which can then further contribute to alpha synuclein aggregation (19). Mitochondrial dysfunction results in ROS generation that can lend an OS environment, and this OS can then further cause mitochondrial damage (20). Still, OS is involved in most parts of the PD disease process, and seems feasible as a primary event that could initiate alpha synuclein aggregation and other

aspects of PD pathology, or at the very least contribute to disease susceptibility. A 2010 study showed that low doses of rotenone delivered intragastrically over long periods of time could generate alpha-synuclein aggregates in neurons that spread from the vagus nerve up to the SN in a mouse model (16). In this study, it is important to consider that rotenone, a mitochondrial inhibitor and potent inducer of OS, started at the intestinal lumen before diffusing into cells and somehow initiating alpha synuclein aggregation in neurons (16). There are no neurons directly lining the intestinal lumen (15).

Though intestinal epithelial cells themselves are an unlikely candidate for initiators of PD pathology due to their fast turnover time, enteroendocrine cells (EECs) are an attractive choice (15). These cells are sparsely distributed throughout the intestinal tract, and line the intestinal lumen just like intestinal epithelial cells, and so are exposed to whatever environment is present there (15). EECs also have a much longer turnover time compared to intestinal epithelial cells, which is important because OS may take time to initiate alpha synuclein aggregation (15). Importantly, EECs express alpha synuclein (15). Lastly, EECs directly contact neurons, which is an essential feature if they are expected to initiate PD pathology that eventually spreads to the brain (15). Once alpha synuclein aggregates reach enteric neurons, it has already been established that alpha synuclein can spread up the vagus nerve to the brain (16). What is currently not known is how aggregates can get to enteric neurons from inside the intestinal lumen.

As a prominent generator of OS that is highly expressed in intestinal epithelial cells, and a protein that has been previously implicated in PD, Nox1 seems like a plausible contributor to intestinal PD pathology (21). The Nox family of NADPH oxidases were among the first enzymes

discovered that generate ROS "intentionally," and not simply as a byproduct of some other process like ATP production in mitochondria (21). Nox2 was the first isoform identified, where it was found to play a role in the respiratory burst and pathogen destruction by phagocytes (21). Today, there are a variety of Nox enzymes known with numerous roles in various tissues (21). Of the several Nox isoforms, Nox1 is highly expressed in the colon, where it was originally cloned (22). Namely, Nox1 is present in colon epithelial cells, and it confers these cells with the ability to produce superoxide (see Figure 1) (22). The role of superoxide generation in these intestinal epithelial cells is thought to either facilitate cell proliferation or act as part of the host response (22). With respect to the host response hypothesis, Nox1 expression is correlated with areas of increased bacterial colonization, and its expression is increased by exposure to interferon-gamma, lipopolysaccharide, and flagellins (22). Moreover, it was found that intestinal epithelial cells rapidly respond to commensal or pathogenic microbes with ROS, likely through a Nox1/Duox2 mediated pathway since these enzymes are highly expressed in intestinal epithelial cells (12).

With respect to PD, Nox1 in the brain was implicated in PD pathology in a number of studies by our lab (23, 24, 25). In a rat 6-hydroxydopamine (6-OHDA) PD model, Nox1 expression was shown to increase in the SN along with DNA oxidative stress (23). In a second study, Nox1 expression was found to increase in a paraquat (PQ) mouse model of PD, and Nox1 inhibition protected Tyrosine Hydroxylase-positive neurons from death in the SN (24). Last, Nox1 knockdown in a PQ rat model of PD was sufficient to reduce Tyrosine Hydroxylase-positive neuron cell death, OS, and alpha synuclein oligomers in the SN (25).

Finally, we investigated high fat diet (HFD) as a possible trigger for PD-related pathology in the gut. First, one study showed that PD patients have an increase in their overall caloric intake, with the most major change occurring with intake of fats (26). Next, in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, mice fed a HFD had lower dopamine levels after treatment and more striatal neuron loss (27). In another study it was shown that mouse fed a HFD prior to being subject to a 6-OHDA model of PD had increased dopaminergic (DA) cell loss as well as increased oxidative stress in the SN (28). Fat intake on the whole has also been show to increase the amount of oxidative stress in the gut (29). Moreover, long-term ingestion of HFD leads to changes in the microbiome (30). A major study related the microbiome to PD by showing that, in an alpha synuclein overexpression model of PD in mice, PD motor symptoms were largely attenuated in germ-free mice (31). Additionally, the study found that microbiome transplant from PD-affected human donors exacerbated physical symptoms when compared to transplant from healthy donors in the overexpression mouse model (31). An overall schematic showing the proposed relationship between an intestinal trigger like HFD, Nox1, OS, and alpha synuclein aggregation is shown in Figure 2.

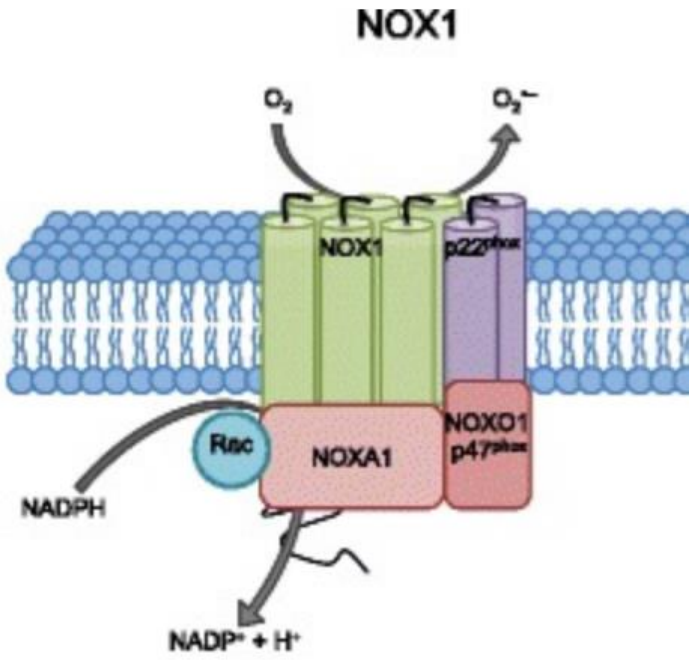


Figure 1: Depiction of Nox1 protein generating superoxide while oxidizing NADPH to $NADP^+$. Also shown are the many subunits associated with Nox1. Cytosolic subunits NOXA1 and NOXO1 are required for superoxide generation. Image from Ma et al, *Molecular Neurodegeneration*

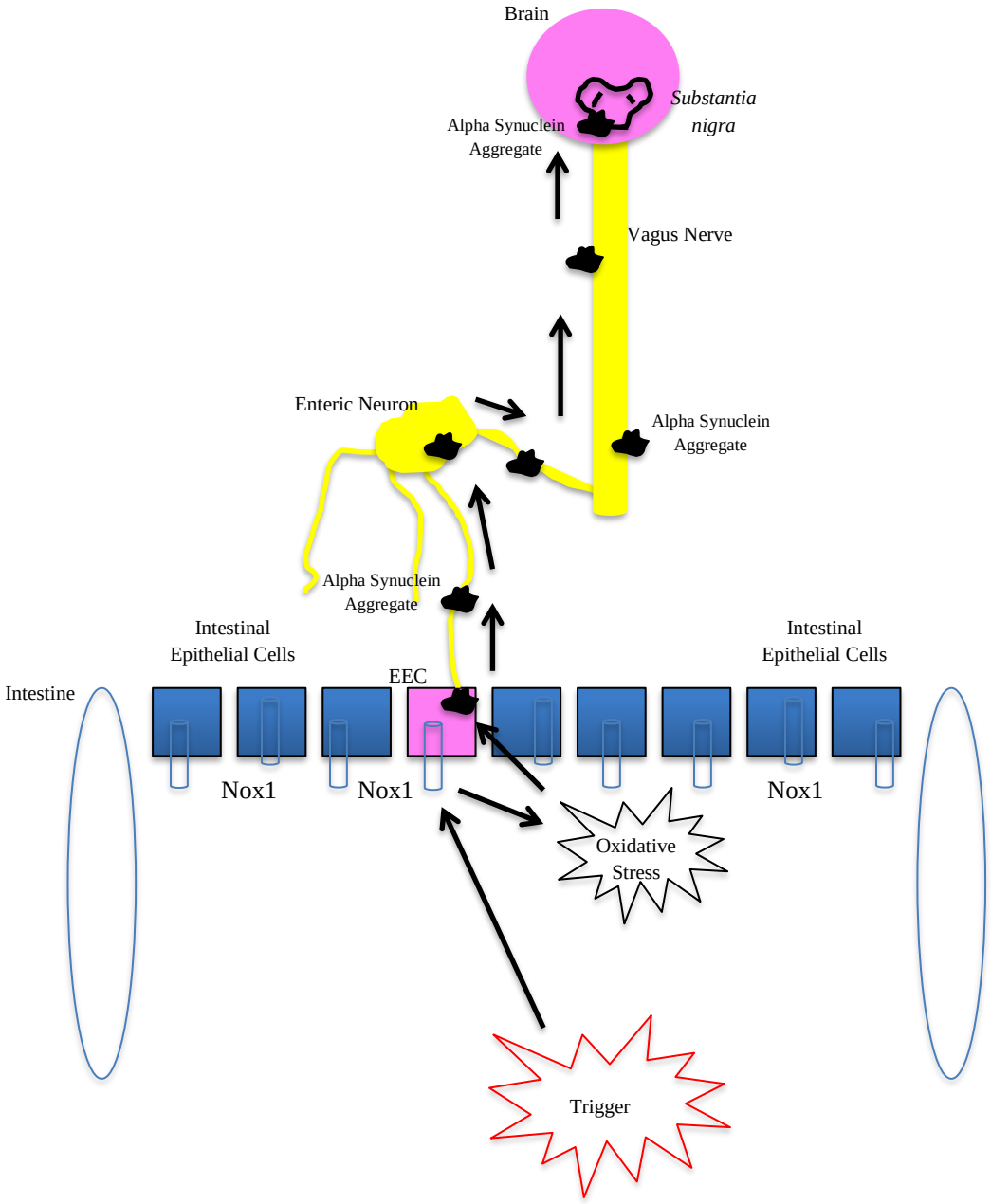


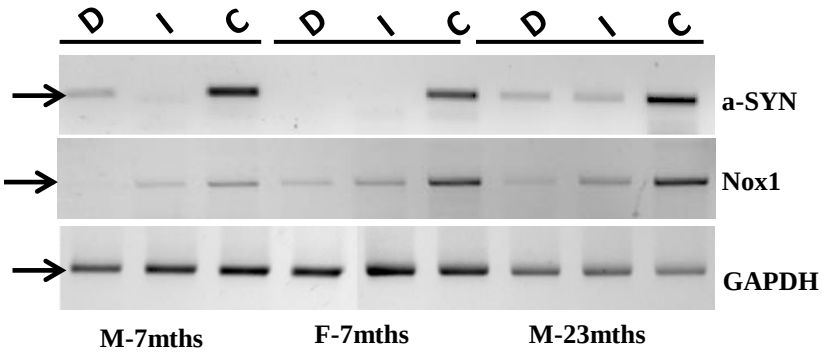
Figure 1: Schematic depicting proposed relationship between intestinal trigger, Nox1, OS, and alpha synuclein aggregation. Intestinal changes initiated by trigger cause EECs contacting the lumen to upregulate expression of Nox1. OS is generated as a result, initiating alpha synuclein aggregation within the EECs. Alpha synuclein aggregates spread from the EECs to enteric neurons contacting the EECs. From there, aggregates spread to the vagus nerve and finally to the *substantia nigra*.

RESULTS

Nox1 and Alpha Synuclein Transcripts are Present in Young and Aged Mouse Colon

Duodenum, ileum and colon tissues were collected from a 7-month old male mouse, a 7-month old female mouse, and a 23-month old male mouse. After that, RNA was extracted using Trizol. cDNA was then synthesized from the RNA, and a Polymerase Chain Reaction (PCR) using the cDNA as template was carried out. Primers for Nox1, alpha synuclein, and GAPDH were used (Figure 3).

This experiment was performed because age is the most significant risk factor for PD, and therefore the presence of Nox1 in aged mice would be helpful in building our hypothesis (32). In addition, oxidative stress is known to increase with age, and an upregulation in Nox1 expression was shown to occur in response to a toxin that induces oxidative stress in the brain in a previous study by our lab (32, 25). This result suggests that Nox1 is present throughout the intestinal tract in mice of both 7 and 23 months of age. It also suggests that alpha synuclein is present in at least the colons of male and female mice of both 7 months and 23 months of age, but it is not consistently detectable in the ileum and duodenum of 7-month old mice.



- **D = Duodenum**
- **I = Ileum**
- **C = Colon**

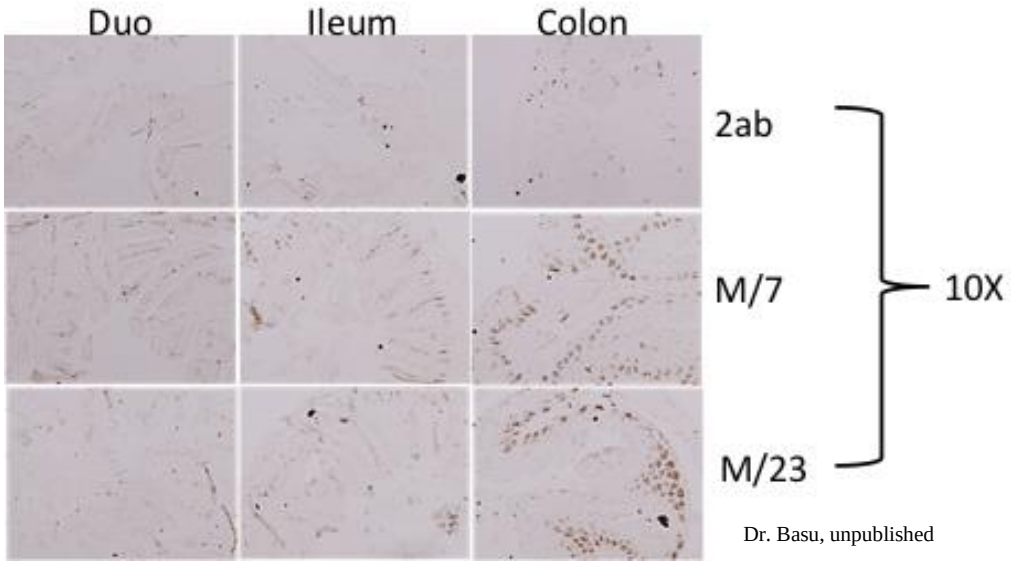
Dr. Basu, unpublished

Figure 3: PCR data from cDNA of Duodenum, Ileum, and Colon of 7-month old male mouse, 7-month old female mouse, and 23-month old male mouse.

Nox1 Protein is Present throughout the Intestines of Young and Aged Mice

Intestinal tissues from duodenum, ileum, and colon were extracted from a 7-month old male mouse and a 23-month old male mouse. Immunohistochemistry (IHC) was then performed on these tissues using Nox1 as the primary antibody. A secondary antibody control, in which only secondary antibody and no primary antibody was used, was performed to establish background staining. Following IHC, 5x, 10x, and 40x images were taken of the tissues. Representative 10x images are shown below (Figure 4).

The expectation was that Nox1 protein levels would be present throughout the intestinal tract, but that Nox1 expression would be highest in the colon. Moreover, we were expecting to see that Nox1 levels would be significantly greater in the colon of the aged mouse when compared to the young mouse. We were able to confirm that Nox1 is in fact expressed thoughout the intestinal tract of both young and aged mice, but it is unclear from this study if there is increased expression in the colon or in the intestines of aged mice.



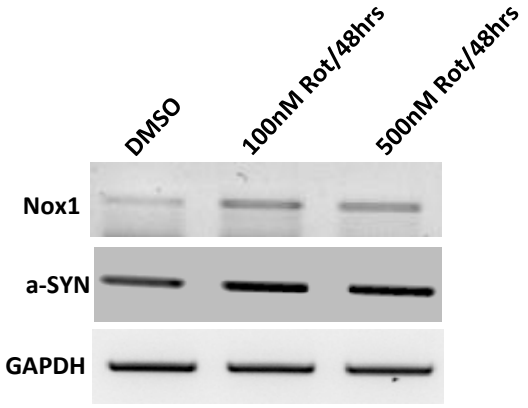
Dr. Basu, unpublished

Figure 4: IHC of Duodenum, Ileum, and Colon from a 7-month old male mouse mouse and a 23-month old male mouse. "2ab" indicates that only secondary antibody was used, which acted as a control for background staining.

STC-1 Cells Express Alpha Synuclein and Nox1 Transcripts

STC-1 cells were treated with either Dimethyl sulfoxide (DMSO) (vehicle control), 100nM of Rotenone or 500nM of Rotenone and collected after 48 hours. The cells then underwent RNA extraction, and cDNA was subsequently synthesized from the RNA. Finally, the cDNA was amplified via PCR using Nox1, alpha synuclein, and GAPDH primer sets (Figure 5).

In a previous study by our lab, it was shown that N27 cells (rat dopaminergic neuron cell line) treated under similar conditions exhibited an increase in Nox1 and alpha synuclein transcript expression (25). It was unclear in the literature whether or not STC-1 cells express Nox1 transcripts. In this experiment, it appears that they do. We also observed in this trial that STC-1 cells express alpha synuclein transcripts, as had been previously reported.



Dr. Basu, unpublished

Figure 5: PCR of alpha synuclein and Nox1 cDNA in STC-1 cells under DMSO, 100nM Rotenone and 500nM Rotenone after 48 hours.

Nox1 Protein Levels are Unchanged in the Intestinal Tissue of Young Mice Fed a HFD

Seven mice fed a HFD for 24 weeks were compared to six age-matched mice fed a normal chow diet. Intestinal tissue was collected at the end of the 24 weeks and paraffin-embedded. Tissue was then sliced using a microtome and dried onto a microscope slide to be used in IHC staining. Nox1 antibody was used as the primary antibody in this study. 5x, 10x, and 40x images were taken of DAB-stained slides following IHC. Representative 10x images from three control and three HFD mouse tissues are shown below (Figure 3). Image quantification was performed using ImageJ on the 10x images for each tissue.

In this case, HFD was investigated as a possible trigger that could enhance Nox1 protein expression in a Nox1-mediated PD model. If HFD did act as a trigger, it would be expected that a significant increase in Nox1 expression could be observed, especially in the colon, where Nox1 protein levels are the highest. It does not appear here that there was any change in Nox1 protein level expression in HFD intestinal tissues compared to controls.

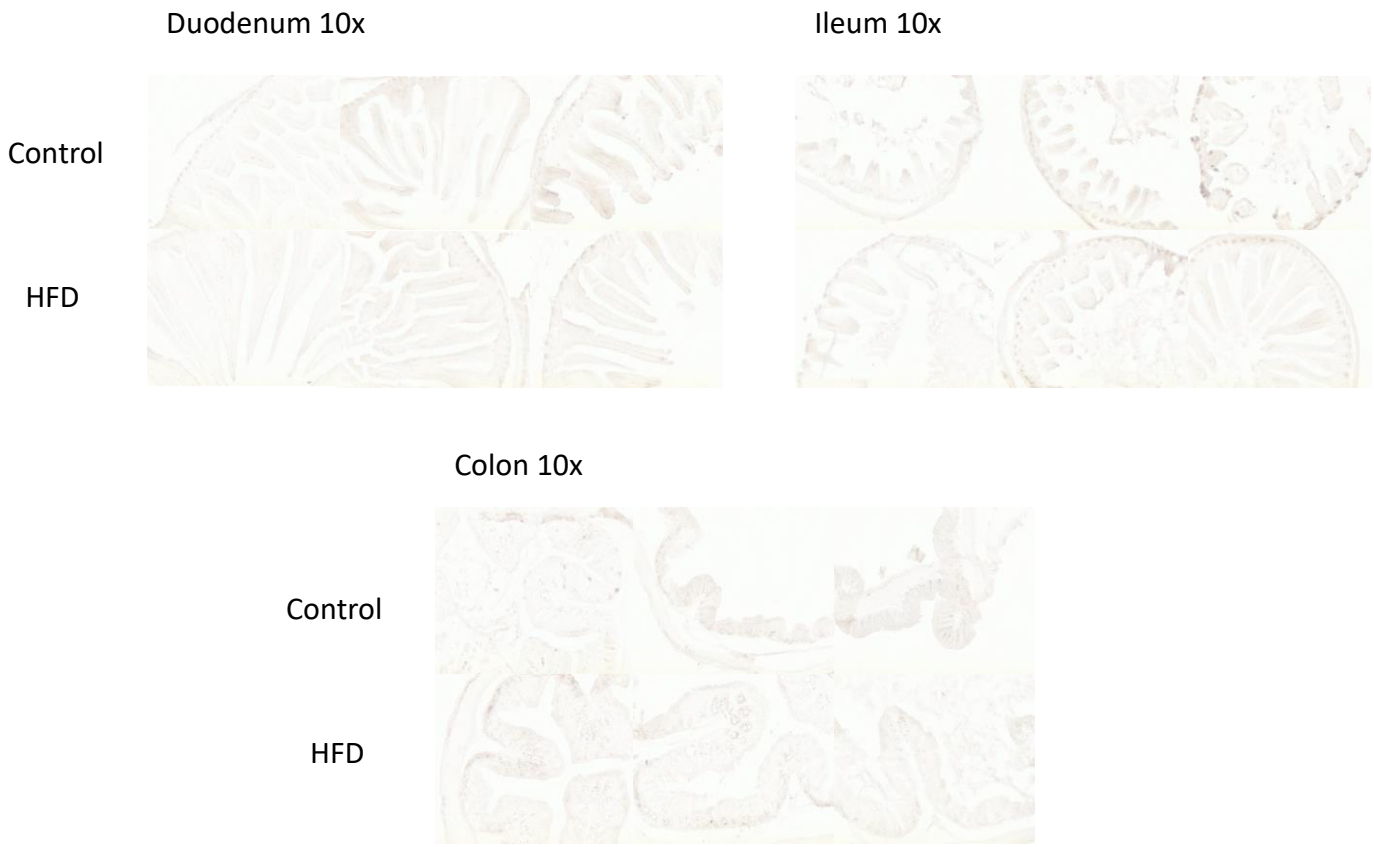


Figure 6: Representative micrographs from Nox1 staining of the intestinal tissues of three animals each in either a HFD or Control diet. Duodenum (top left), ileum (top right), and colon (center) sections are shown with animals in the Control diet group in the top row and animals in the HFD group in the bottom row.

MATERIALS AND METHODS

STC-1 Cell Culture Maintenance

STC-1 cells (ATCC) were maintained above five passages in DMEM high glucose supplemented with 20% FBS. Cell splitting was achieved by first incubating cells with Trypsin-EDTA (TE) for 1-2 minutes, inactivating the TE by addition of equal volume of culture media, and pelleting the cells by 5 minutes of centrifugation at 500xg. The supernatant was then aspirated and the pellet resuspended in 1ml of media. This cell suspension was then aliquoted in appropriate volumes to new culture dishes or the same dish for the desired amount of cell splitting.

RNA Extraction

RNA extraction was performed using 500ul of Trizol reagent (Invitrogen) for STC-1 cell pellets and 1ml Trizol reagent for tissue samples. Cell pellets and tissue samples were homogenized by pipetting up and down using a p1000. A p200 was used if further dissociation was required. For the tissue samples, even after pipetting and prolonged incubation in Trizol, some material did not dissolve. In this case, samples were spun down as 12,000xg for 10 minutes at 4°C, and the supernatant was collected and transferred to a new 1.5ml tube. After that, the RNA extraction proceeded as described in the Invitrogen protocol. Following extraction, the RNA pellet was allowed to dry in a 1.5ml tube with the lid open for 20 minutes. Samples were resuspended in 30ul to 50ul DEPC-treated water depending on pellet size.

cDNA Synthesis

cDNA synthesis consisted of using the GenDEPOT amfiRivert cDNA Synthesis Platinum Master Mix. A mixture was prepared consisting of 1ug of RNA brought up to a volume of 9ul with DEPC water, 10ul of a 2X buffer, and 1ul of reverse transcriptase enzyme. The reaction mixture was then incubated in a thermocycler at the following conditions: 1 minute at 60°C, 5 minutes at 25°C, 1 hour at 50°C, and 1 minute at 85°C.

Polymerase Chain Reaction

PCR was carried out using the GenDEPOT amfiSure PCR Master Mix (2X). The total reaction volume used was 16ul, and it contained 8ul of the 2X Master Mix, 1ul of 10uM forward primer, 1ul of 10uM reverse primer, 1ul of template DNA and 5ul of PCR-certified water. Following amplification, DNA amplicons were analyzed via agarose gel electrophoresis. A 1.2% agarose gel with 0.5ug ethidium bromide per 1ml agarose was typically used, and the gel was run at 100V for approximately 30 minutes. Depending on the amount of samples involved, gels were either prepared to a volume of 75ml or 150ml. Following electrophoresis, agarose gels were imaged in a Gel Doc Easy Imager (Biorad) with 0.2 seconds of light exposure.

Immunohistochemistry

Paraffin-embedded tissues were cut to 25 micron slices using a microtome and then placed on microscope slides. Tissue slices on slides were allowed to dry overnight. After that, slides were deparaffinized in 2 changes of xylene for 5 minutes each, followed by incubation in

2 changes of 100% ethanol for 3 minutes each, 95% ethanol for 1 minute, and 80% ethanol for 1 minute. All slides were then boiled at 95-100°C in Sodium Citrate Buffer (10mM Citric Acid, 0.05% Tween 20, pH 6) for 30 minutes. The slides were then allowed to cool for 20 minutes, and then rinsed twice in PBS-T (PBS with 0.05% Tween 20) for 5 minutes each. Endogenous peroxidase blocking was achieved by incubating slides in 3% hydrogen peroxide in PBS for 5 minutes. Slides were rinsed in PBS-T and then permeabilized in 0.2% Triton in PBS. Next, blocking was performed in a solution of 1% Bovine Serum Albumin (BSA) and 5% horse serum in PBS-T for 30 minutes. Samples were washed in PBS-T and then incubated in primary antibody overnight at 4°C in a humidifier. Primary antibody staining was performed using a 1:25 dilution of Nox1 antibody serum (a gift from the lab of Yabe-Nishimura). The next day, samples were washed three times in PBS-T and secondary antibody coupled to biotin was applied for one hour. Slides were washed again with PBS-T and then slides were incubated in the Avidin-Biotin Complex Reagents (Vecta Stain) for 1 hour. The samples were washed in 0.1M PB and then DAB stain (0.7mg/1mL DAB in a solution of 30% hydrogen peroxide and 0.1M PB) was applied for 2 minutes to each samples, in set of three slides at a time. After that, all slides were dehydrated by immersing in dH_2O for 5 minutes, 70% ethanol for 5 minutes, 80% ethanol for 5 minutes, 90% ethanol for 5 minutes, and 2 changes of 100% ethanol or 5 minutes. The samples were then placed in xylene for 15 minutes and mounted on a coverslip using Permount. The coverslips were allowed to dry overnight before being visualized on a Leica microscope.

Aged Mouse Studies

Wild Type (WT) C57BL/6 mice were used in these studies, including a 7-month old male mouse, a 7-month old female mouse, and a 23-month old male mouse.

High Fat Diet Treatment

WT C57BL/6 mice were fed a 60% HFD for 24 weeks. All mice were 32 weeks (8 months) old. Body weight and food intake for each mouse was monitored on a weekly basis. The control group in the study consisted of three females and three males, and the HFD group consisted of four females and three males.

DISCUSSION

It is difficult to come to any concrete conclusions with the data obtained here, as much of it needs to be repeated. However, the data seems to suggest that Nox1 transcripts and protein are in fact present throughout young and aged mouse colons. It also seems that alpha synuclein transcripts exist in STC-1 cells. Also in STC-1 cells is the suggestion that Nox1 transcripts exists. If this observation is proven to be true after repeated experiments, it would simplify our downstream *in vitro* experiments in that a co-culture of STC-1 cells and intestinal epithelial cells would not be necessary. Finally, feeding mice a HFD does not appear to affect Nox1 protein levels in the intestinal tract. To confirm this, it may be necessary to further explore the functionality of ImageJ and find a way to measure staining in specific structures, as opposed to measuring overall staining.

The data shown here represent only the very beginning steps of a long study involving Nox1's role as a mediator of gut pathology. As stated above, many more repeats of the data shown here will be needed. Also down the road, larger cohorts will be necessary to generate findings that are meaningful. Moreover, other experiments may be used, including western blots and qPCR, to evaluate differences in transcript and protein expression.

Finally, in the future, we may continue to investigate HFD as a possible trigger for Nox1-mediated PD pathology in the gut, despite its apparent lack of a connection to Nox1 in this initial study. More focus, however, will be devoted to investigating *P. mirabilis* as a possible mediator of gut pathology, based on the study by Choi et al (34).

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