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RUSH UNIVERSITY MEDICAL CENTER

Targeting Diet-Microbiome Interactions in the Pathogenesis of Parkinson's Disease

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BACKGROUND/RATIONALE

Summary. The microbiome influences neurodevelopment, modulates behavior, and contributes to various neurological and neuropsychiatric disorders. Parkinson's disease (PD) affects 1% of the US population over the age of 60, resulting in cognitive and motor dysfunction, and is characterized by aggregation of α-synuclein (αSyn). While the etiology of PD is largely unknown, environmental factors appear to play a role in most cases. Our team has recently discovered that the microbiome promotes motor deficits, induces microglia activation, and increases αSyn pathology in a mouse model of PD (Sampson et al., *Cell*, 2016, in press). Antibiotic treatment ameliorates, while microbial colonization enhances, pathophysiology in adult animals, suggesting symptoms arise from postnatal signaling between the microbiota and the brain. We tested whether metabolites produced by gut bacteria from food impact disease, a notion supported by epidemiological data that fiber content in diet affects motor symptoms in PD patients. Indeed, we show that feeding short-chain fatty acids (SCFAs), microbial fermentation products of dietary fiber, modulates neuroinflammation, αSyn aggregation in the brain, and motor symptoms. These findings reveal that gut bacteria impact disease outcomes in a mouse model via modulation of microbial metabolites produced from food. The current project will analyze the gut microbiome and metabolites from PD patients and controls, and employ clinically relevant mouse models to determine how metabolites produced by the microbiome from dietary substrates affect motor symptoms. Finally, we propose to test whether directly regulating microbial metabolite profiles using "designer" dietary fibers and probiotics offers new avenues for ameliorating PD-like symptoms.

HYPOTHESIS

Interactions between diet and the microbiome contribute to the pathophysiology of PD, and targeted modulation of dietary metabolites produced by the microbiome can ameliorate disease course and/or symptoms

OBJECTIVES

Studies have correlated diets, which in turn affect microbiota composition, with both positive and negative PD outcomes [1]. However, mechanisms of action have not been identified. Recent advances describing how the microbiome critically impacts the immune, metabolic and nervous systems may help provide an explanation to the link between diet and PD [2-6]. Gut bacteria process dietary components to produce microbial metabolites, which enter the host and access almost every tissue of the body, including the brain [5-7]. We propose the novel hypothesis that variations in gut microbiome composition alter the metabolic output of ingested dietary fibers, modulating SCFA profiles that impact pathophysiology and motor deficits in PD. Our project objectives are: **1**) define a disease-modifying role for the human gut microbiome in PD, **2**) determine how the microbiome and its metabolites impact disease, and **3**) test "designer" diets and probiotics that ameliorate symptoms in mice. If successful, this research will support the transformative concept that the gut microbiome contributes to the pathogenesis of PD, and may advance research toward development of dietary and probiotic therapies.

Parkinson's disease. Behavioral, psychiatric and neurodegenerative disorders often display hallmark neuropathologies within the central nervous system (CNS). One neuropathology, amyloidosis, results from aberrant aggregation of specific neuronal proteins that disrupt many cellular functions. Affected tissues often contain insoluble aggregates of proteins with altered conformations, a feature believed to contribute to an estimated 50 distinct human diseases [8]. Neurodegenerative amyloid disorders, including Alzheimer's, Huntington's, and Parkinson's diseases, are each associated with a distinct amyloid protein [9]. PD patients display motor deficits including tremors, muscle rigidity, bradykinesia, and impaired gait. It is a multifactorial disorder that has a strong environmental component, as less than 10% of cases are hereditary [10]. Aggregation of α-synuclein (αSyn) is thought to be pathogenic in a family of diseases termed synucleinopathies, which includes PD, Multiple System Atrophy (MSA), and Lewy body Dementia [9, 11, 12]. αSyn aggregation is a stepwise process, leading to oligomeric species and intransient fibrils that accumulate within neurons. Dopaminergic neurons of the substantia nigra pars compacta (SNpc) appear particularly vulnerable to the effects of αSyn aggregates. Dopamine modulators are a first line therapeutic in PD; however treatments can carry serious side effects and often lose effectiveness [13]. Discovery of safe and effective therapeutics are needed to address the increasing burden of PD in an ever-aging population, a paradoxical consequence of mankind's achievements in increased lifespan.

The gut-brain axis. Although neurological diseases have been historically studied within the CNS, peripheral influences are implicated in the onset and/or progression of diseases that impact the brain [14]. While gut-brain interactions have been appreciated for many decades, providing a wealth of information about the close

interactions between the gut associated immune system, enteric nervous system (ENS) and gut-based endocrine system [15], these findings have largely been ignored by the neuroscience community. Recently, emerging data suggest bidirectional communication between the gut and the brain in anxiety, depression, nociception and autism spectrum disorder (ASD), among others [6, 16, 17] Gastrointestinal (GI) physiology is influenced by signals arising both locally within the gut and from the CNS. Neurotransmitters, immune signaling, hormones and neuropeptides produced within the gut may, in turn, impact the brain [18, 19]. Research into how the gut-brain axis influences neurological conditions may reveal insights into the etiology of some CNS disorders.

The microbiome and the nervous system. The human body is permanently colonized by microbes on environmentally exposed surfaces, the majority of which reside within the GI tract [20]. Mounting evidence over the past decade has suggested the gut microbiome critically controls the development and function of the immune and metabolic systems [2, 3]. Increasingly, new research is beginning to uncover the profound impacts that the microbiota can have on neurodevelopment and the CNS [6]. Germ-free (GF) mice and antibiotic treated specific pathogen free (SPF) mice are altered in hippocampal neurogenesis and display impaired spatial and object recognition [21]. Gut bacteria affect expression of the serotonin (5-hydroxytryptamine; 5-HT) receptor, brain-derived neurotropic factor (BDNF), and NMDA receptor subunit 2 (NR2A) [22-24]. GF mice have altered cortical myelination and impaired blood-brain barrier function [25, 26]. Additionally, the microbiota promotes enteric and circulating serotonin production in mice [27], and affects anxiety, hyperactivity and cognition [19, 22, 28, 29]. Adding relevance to these findings, dysbiosis (i.e., alteration) of the human microbiome has been reported in subjects diagnosed with several neurological diseases [17]. For example, fecal and mucosa-associated gut microbes are different between individuals with PD and healthy controls [30-33]. Yet, how dysbiosis arises and whether this feature contributes to PD pathogenesis remains unknown.

Gut bacteria influence the differentiation and function of immune cells in the intestine, periphery and brain [34-36]. Intriguingly, subjects with PD exhibit intestinal inflammation [37], and GI abnormalities such as constipation often precede motor symptoms by many years [38, 39]. Braak's hypothesis posits that aberrant αSyn accumulation initiates in the gut and propagates via the vagus nerve to the brain [40]. This notion is supported by pathophysiologic evidence: αSyn inclusions appear early in the ENS, and the glossopharyngeal and vagal nerves [38, 41], while vagotomized people are at reduced risk for PD [42]. Further, injection of αSyn fibrils into gut tissue of healthy rodents induces pathology within the vagus nerve and brainstem [43]. However, the notion that αSyn aggregation initiates in the ENS and spreads to the CNS via retrograde transmission remains controversial [44], and experimental support for a gut microbial connection to PD is lacking.

Gut bacteria and diet. Microbiota composition and function are shaped over the course of an individual's life and depend on many factors including a significant role for diet [4]. In humans and mice, both long-term dietary patterns [45] and rapid, extreme dietary changes [46] can shape and re-shape the representation of microbial taxa and their functional attributes. Since many of the precursor molecules available for microbial metabolism are provided by diet, altering diet can alter the metabolic output of the microbiome. Broad effects of diet on the microbiome and its associated metabolome were observed when humanized mice were switched from standard mouse diets to a high sugar, polysaccharide-deficient diet [47]. Although mice harboring microbiotas from three different humans showed distinct composition, metabolomic fingerprints of the three groups were remarkably similar when fed the same diet [47], illustrating conservation of microbial metabolism. The principle of dietary impacts to the metabolic output of the microbiome has also been established in humans [48].

Diet and Parkinson disease. Epidemiological studies have shown a robust relationship between indices of nutritional health and PD. Constipation is associated with an increased risk of PD [49], with loss of dopamine transporter upon imaging [50] and with αSyn pathology and loss of midbrain dopamine neurons [51, 52] that are typical of central nervous pathology in PD. Mid-life adiposity is also predictive of the development of PD [53], and risk of the disease is increased in subjects with low dietary intake of polyunsaturated fatty acids, flavonoids [54] vegetables, fruits and nuts [55-57]. Thus, most relevant to our hypothesis, it appears that patients with PD consume diets low in fiber compared to the controls.

Many recent studies have focused on intestinal bacterial metabolites that may affect brain function and neuroinflammation [58-61]. Among these microbiota-produced metabolites, perhaps short chain fatty acids (SCFAs) have received the most attention. SCFAs are the short (C1-C4) saturated fatty acids formate, valerate, acetate, propionate and butyrate that are formed by intestinal bacteria as products from the fermentation of non-

digestible carbohydrates, mostly in the colon [62, 63]. Broadly speaking, SCFAs can affect neuroinflammation

through two distinct mechanisms: 1) regulation of inflammation via SCFA receptors [64-66]; and 2) epigenetic regulation of neural function and neuroinflammation through histone deacetylase inhibition (HDACi) [67, 68].

With regard to SCFA receptors, acetate, propionate and butyrate have been shown to bind to specific receptors on mammalian cells including Gpr41, Gpr43, and Gpr109a [69]. Also, the transport proteins MCT1 and Slc5A8 are required for SCFAs to enter cells and exert HDACi activity [69]. Gpr41 preferentially binds to propionate and butyrate. Gpr43 is primarily activated by acetate and propionate, and Gpr109a is specific for butyrate [69]. Signaling via Gpr109a is associated with modulation of inflammation [70]. Significantly, Gpr109a was recently shown to be upregulated on microglia in the substantia nigra of PD patients [71]. As a result, Gpr109a has been suggested as a treatment target for PD [72]. However, our knowledge of how SCFAs and their receptors influence brain function and potentially participate in CNS pathologies like PD is still limited and requires further studies [67].

Some of the most compelling evidence for the ability of SCFAs to impact neuroinflammation in PD comes from epigenetic studies that show the HDAC inhibitory activity (HDACi) of butyrate. PD appears to be characterized by global histone deacetylation in the brain [73]. Significantly, αSyn exerts HDAC activity by binding directly to histone H3 and promoting deacetylation and neurotoxicity that can be reversed with HDACi such as butyrate [74]. In support of this notion, butyrate and other HDACi have been shown to suppress pathology and neuroinflammation in multiple models of PD [75]. The novel HDACi K560 protected against neuroinflammation and pathology in both the MPP+ and MPTP models of PD in mice [76]. HDACi also protect from neuroinflammation and pathology in the rat rotenone model of PD [77]. Consistent with these above data for the protective activity of butyrate in PD is our microbiome profiling data showing decreased numbers of butyrate producing bacteria in PD patients [30]. Our finding was recently replicated by others who also showed significantly decreased fecal SCFA levels in PD patients [78]. Therefore our strategy of studying the role of SCFAs in the pathogenesis of PD and our approach to modulate butyrate production by the intestinal microbiota through prebiotic fiber feeding as a therapy for PD is based on strong scientific premise.

SPECIFIC AIMS

Specific aim 1. Profile the human stool microbiome, and serum and stool SCFA levels, in early PD patients. We will profile the human fecal microbiome and metagenome from new onset PD patients and controls by next generation shotgun sequencing and bioinformatics analysis, and determine SCFA levels and ratios in stool and serum. By correlating disease severity scores with specific microbial metabolites, we will determine whether SCFA levels and/or ratios are a biomarker for PD, and identify candidate microbiota from new PD patients and healthy controls for transplant into germ-free (GF) mice for functional studies.

Specific aim 2. Determine how the microbiome regulates gut-brain interactions in a PD model. We will transplant human microbiota into germ-free mice from high (top 3rd) and low (bottom 3rd) SCFA producers, from PD and HC donors identified in Aim 1. We will assess motor symptoms, gut motility, neuroinflammation and αSyn aggregation. Advanced tissue clearing approaches (CLARITY) will be used to image changes in gut and brain neuronal circuits, and measure neuronal activation and electrophysiology in intestines and brains of "humanized" mice. These studies will reveal functional links between the microbiome and the pathophysiology of PD by determining gut-brain connections in a novel and relevant mouse model.

Specific aim 3. Develop and test "designer" fiber diets to modulate SCFA levels in PD mice. SCFAs are a collection of dietary fermentative breakdown products that impact the immune and nervous systems. We have developed a novel library of fiber diets, and validated in bioreactors that adding specific dietary mixtures to microbial communities can shape the SCFA repertoire. We will test dietary fiber mixtures that produce "healthy" SCFA ratios from PD stools *in vitro*, and treat mice "humanized" with a PD microbiota to regulate SCFA levels. Neuroinflammation, αSyn pathology, and motor symptoms will be assessed. These studies aim to modulate disease outcomes through rational dietary control of microbiomes and their metabolites.

Specific aim 4. Test dietary and probiotic treatments in mice with PD-like symptoms. We predict that the efficiency of a microbiome to utilize dietary substrates varies based on its composition, and an optimal intervention may require modulating both diet and the microbiome. Further, different SCFA profiles likely alter gut bacterial composition. Herein, we will mine microbiome sequence data to identify bacteria that are selectively reduced or missing in PD patients, and treat humanized PD mice with these potential probiotics.

Finally, we will feed specific diets that shape SCFAs ratios and administer candidate bacteria simultaneously to humanized mice with PD symptoms, to test potential combinatorial (i.e., prebiotic and probiotic) interventions.

RESEARCH STRATEGY

Preliminary data. The Thy1-α-synuclein (ASO; alpha-synuclein overexpressing) mouse model displays progressive deficits in motor function similar to human PD. ASO animals harboring a complex microbiota (SPF-ASO) require significantly more time to walk across a challenging beam compared to wild-type (WT) littermates (SPF-WT), and also exhibit increased time to descend from the top of a pole to its base, two measures of gross motor function (**Figures 1A, B**). In addition, removal of an adhesive from the nasal bridge, a test of fine motor control, is impaired in SPF-ASO mice (**Figure 1C**). Finally, the hind limb clasping reflex, a measure of striatal dysfunction [79, 80], is also defective in ASO mice (**Figure 1D**). To assess a contribution of gut bacteria, we re-derived ASO mice under germ-free conditions (GF-ASO) and compared performance in these tests to tests. Remarkably, GF-ASO animals exhibit reduced deficits in the time to cross the beam, descend a pole, remove a sticker, and display improved hind limb clasping (**Figures 1A-D**).

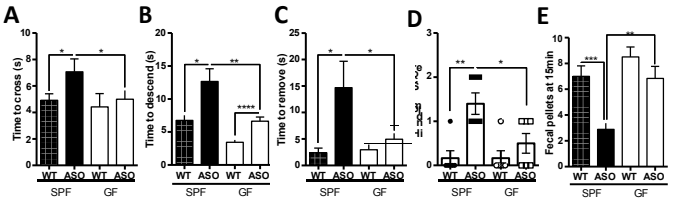


Figure 1. Gut microbes promote motor and gastrointestinal (GI) dysfunction. (A) Time to traverse beam apparatus. **(B)** Time to descend pole. **(C)** Time to remove adhesive from nasal bridge. **(D)** Hind-limb clasping reflex score. **(E)** Total fecal pellets produced in 15 minutes. Animals tested at 12-13 weeks of age. N=4-6, error bars represent mean from 3 trials per animal. Data representative of 2 repeats per experiment. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. SPF=specific pathogen free, GF=germ-free, WT=wild-type, ASO=Thy1-α-synuclein.

Subjects with PD exhibit intestinal inflammation [37], and GI abnormalities such as constipation often precede motor defects by years [38, 39, 41, 81], leading to the intriguing hypothesis that PD may originate in the gut [38]. GF animals display significantly improved fecal output compared to mice harboring gut bacteria, revealing that the microbiota enhances GI motility in ASO mice (**Figures 1E**). Further, stool from SPF-ASO mice contain reduced water content compared to GF-ASO mice (data not shown). Together, these findings demonstrate that gut microbes modulate the hallmark motor *and* intestinal dysfunction in a preclinical model of PD.

Motor deficits in PD coincide with the accumulation of αSyn into insoluble aggregates [82]. Under SPF conditions, we observe by microscopy of ASO brain tissue notable aggregations of αSyn in the caudoputamen (CP) and substantia nigra (SN) (**Figures 2A and B**), brain regions of the nigrostriatal pathway affected in both mouse models and human PD [9]. Surprisingly, GF-ASO mice display appreciably less αSyn aggregates in these regions (**Figures 2C and D**). αSyn aggregates are immunostimulatory and activate immune cells, including brain-resident microglia [83-85]. Microglia undergo significant morphological changes upon activation, transitioning from thin cell bodies with numerous branched extensions to round, amoeboid cells with fewer processes [34, 86]. Within the CP and SN, microglia of SPF-ASO mice display significant increases in cell body diameter, along with fewer processes of shorter total length, compared to GF-ASO mice (**Figures 2E, F**). Overall, we reveal that the microbiota promotes αSyn aggregation and activation of microglia within specific brain regions linked to PD.

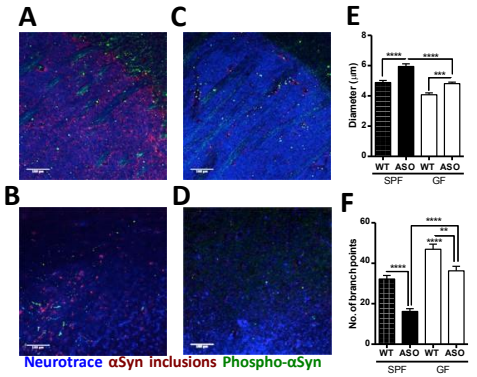


Figure 2. αSyn pathology and microglia activation is increased in mice harbouring a gut microbiota. (A, C) Representative images of the caudoputamen from **(A)** SPF-ASO or **(C)** GF-ASO animals stained with aggregation-specific αSyn antibody (red), Phospho-Ser129-αSyn antibody (green), and Neurotrace/Nissl (blue). **(B, D)** Images of the substantia nigra from **(B)** SPF-ASO or **(D)** GF-ASO animals. Substantia nigra-resident microglia parameters **(E)** diameter and **(F)** number of branch points.

The microbiota influences brain development during gestation, as well as modulates gut-brain signaling in adulthood [5]. To differentiate between these mechanisms, we treated adult SPF animals with an antibiotic cocktail to

deplete the microbiota postnatally. Conversely, we colonized adult GF mice with a complete mouse microbiota. We found that depleting the microbiome with antibiotics ameliorated motor and GI deficits, αSyn pathology and microglia activation in ASO mice (not shown due to space constraints). Colonizing GF mice with gut

bacteria restored PD-like symptoms. Thus, altering gut bacteria during adulthood contributes to αSyn-mediated pathophysiology. This reversibility of symptoms suggests active gut-brain signaling by the microbiota.

Recently, it was revealed that gut bacteria modulate microglia activation or maturation through production of short-chain fatty acids (SCFAs) [34]. Thus, we treated GF animals with a mixture of SCFAs: acetate, propionate, and butyrate (mice remained microbiologically sterile). Within affected brain regions (CP and SN), microglia in SCFA-administered animals display increased activation compared to untreated mice (data not shown). Further, SCFA treatment increased αSyn aggregations in the CP and SN (data not shown). Oral SCFA treatment of GF mice is sufficient to induce αSyn-mediated impairment in beam traversal, pole descent, and hind limb reflex (**Figures 3A-D**; compare GF-ASO to SCFA-ASO). SCFAs promote constipation-like symptoms (**Figure 3E**). We propose that the microbiota actively produces metabolites from dietary fibers that regulate microglia activation and αSyn aggregation, contributing to symptoms in a mouse model of PD.

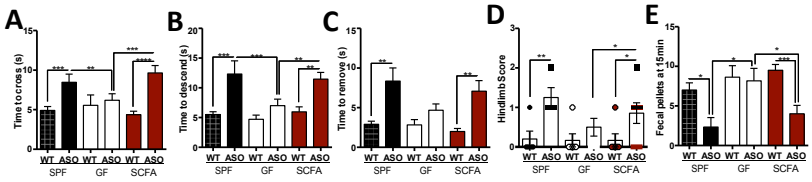


Figure 3. SCFAs promote αSyn-stimulated microglia activation and motor dysfunction. (A) Time to traverse beam apparatus. **(B)** Time to descend pole. **(C)** Time to remove nasal adhesive. **(D)** Hind limb clasping reflex score. **(E)** Total fecal pellets produced in 15 minutes. Microglia activation by SCFA feeding not shown due to space constraints, but see Sampson et al., *Cell* (in press) for data.

Given our observation that postnatal signaling by the microbiota influences motor function in mice, and evidence that PD patients have altered microbiomes [30-32], we sought to determine whether gut microbes from patients with PD impact disease outcomes when transferred into mice. We collected fecal samples from 6 human subjects diagnosed with new onset PD as well as 6 matched healthy controls (HC) at RUMC, and transplanted human microbiota into GF animals to generate "humanized" mice at Caltech. Remarkably, beam traversal, pole descent and hind limb reflex scores are significantly impaired in ASO animals that are colonized controls (**Figure 4**). We will use this powerful experimental approach to define how human gut bacteria regulate neuroinflammation, pathophysiology and motor symptoms.

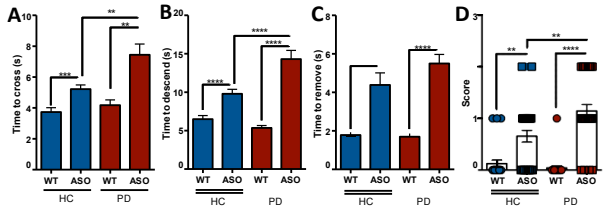


Figure 4. Microbiota from PD patients induce increased αSyn-mediated motor deficits in mice. Compiled, averaged data from all 6 independent cohorts in each motor task described in Figure 3. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$. HC=germ-free mice colonized with fecal microbes from healthy controls, PD=germ-free mice colonized with fecal microbes from Parkinson's disease patients, WT=wild-type, ASO=Thy1-α-synuclein genotype. Note: for all 6 individual pairs, mice with PD microbiota performed worse in most or all tests compared to HCs. Also, we have not excluded any fecal samples tested in this data set, thus these results appear to be robust.

<u>Specific aim 1. Profile the human stool microbiome, and serum and stool SCFA levels, in early PD patients and matched healthy controls</u>

Introduction. We [30] and others [31] have shown that patients with PD have abnormal microbiota compositions. These data support the hypothesis that the human microbiome could either trigger PD or impacts PD disease course. However, several questions remain to be answered: (**1**) what is the exact nature of the dysbiosis in PD and how specific is the dysbiosis for PD compared to other synucleinopathies? (**2**) what are the contributing factors for the observed dysbiosis? and (**3**) how does the dysbiosis promote PD pathogenesis? The objective of this aim is to fill the translational knowledge gap between dysbiosis of the microbiome and PD.

Aim 1.1. <u>Comprehensively interrogate microbiome composition to elucidate the exact nature of the dysbiosis in PD and to determine whether the observed dysbiosis is specific for PD</u>

Rationale. The currently published data on microbiome composition present in PD utilized targeted approaches

such as 16S rRNA pyrosequencing [30, 31]. These techniques provided accurate assessment of microbiota composition to the genus level, but are not adequate to provide species or strain level determination and thus

further studies using next generation sequencing and advanced bioinformatics are needed to comprehensively interrogate microbiome composition. This approach provides very detailed information about the microbiome that may enable better understanding of host-microbial interaction processes, and may identify bacteria that are either missing or over-representing in the PD subjects, possibly providing leads toward identification of bioactive species for study in Aim 4 and future research.

Experimental Approach. We have already collected and biobanked fecal and blood samples from 40 well-characterized (including 20 newly diagnosed, treatment naïve) PD patients and 20 matched [for age, gender, race, BMI] healthy controls (HC). We also have comprehensive dietary information (using 24 hour diet recall questionnaire prior to stool collection) in these subjects. To profile the human fecal microbiome, DNA will be extracted using the NexteraXT sample preparation kit adapted for acoustic liquid handling, and subjected to whole-genome shotgun metagenomic sequencing. This procedure is routine to our team [87-90], but briefly, shotgun metagenomics will be performed using the NexteraXT barcoded kit on the Labcyte Echo and TPP Mosquito platforms, allowing low-biomass inputs. Sequencing will be performed at 2X150 bp using the Illumina HiSeq 4000 platform, allowing excellent depth of coverage, and mapped to ~47,000 reference bacterial genome sequences using HUMAnN2. In our experience, assembly using metaSPADES [91] does not work well for data generated using this method, but when the reference genome collection is fairly complete, as it is for human stool microbiomes, read mapping provides excellent taxonomic and functional results. Bioinformatics analyses using HUMAnN2 and QIIME [92] will map metagenomic DNA data sets to determine gene content and the corresponding microbial pathways involved. Though we will mine this data exhaustively for global multi-parameter analysis, we will specifically determine whether genes associated with known SCFA pathways are enriched or depleted differentially in PD patients and controls.

Our hypothesis is that microbiota-derived metabolites contribute to PD symptoms, and may be protective or pathogenic depending on metabolite levels and/or ratios. The question is whether hallmark changes in microbiome composition and function are specific to PD or are the consequence of aggregated αSyn in the ENS and intestinal dysfunction. To answer this question, we will collect stool samples from patients with Multiple System Atrophy (MSA), a synucleinopathy, and α-synuclein-dementia [n=20 each group], and interrogate their microbiome composition and function using shotgun metagenomics as described. Comparison to the PD microbiome will address specificity to related neurodegenerative disease, and this knowledge could also further expand the role of microbiome to additional disorders where αSyn plays a pathogenic role.

Aim 1.2. Identify the potential contributing factors for dysbiosis in PD

Rationale. It is well established that environmental factors such as diet, as well as genetics, impact microbiome composition [93]. Currently, there are no data for how environment impacts the microbiome in PD. Filling this knowledge gap can advance our understanding of the underlying mechanism of dysbiosis in PD and provide an opportunity for disease modification in those at high risk of developing PD, such as first-degree relatives.

Experimental Approach. In order to determine the contribution of environmental and genetic factors to dysbiosis in PD, we propose two approaches. First, a cross sectional study where we will compare microbiome composition in subsets of PD patients (newly recruited as part of the proposed application) whose household, non-related spouse (for environmental factor) and their first-degree relatives who do not live with the patient agree to provide stool samples as well. The consent process will be initiated after the subject has been initially screened for eligibility. If the patient meets inclusion and exclusion criteria, the subject will be approached by a member of the study staff during his/her regular clinic visit or sent a copy of the consent form so that they may read it in the comfort of their own home. If the potential subjects' spouse/ relative accompany during the clinic visit, they will be informed about the study. If not, the subject will be asked to share the study information with their spouse/ relative and inform the study team if interested. If the subject is interested in moving forward, the consent will be reviewed by the study coordinator in the Gastroenterology clinic. The study coordinator, PI or study staff delegated the responsibility by the PI will obtain informed consent from the subject.

We also plan to have the study registered on clinicaltrials.gov and the Michael J Fox trial finder so that interested participants can contact the study team.

Each household and first-degree relative study subject will be evaluated to ensure there is no clinical evidence of neurological disorders including PD. Also, these subjects will complete a 24 hour diet recall questionnaire before stool collection and validated 3 month food frequency questionnaires to collect dietary information similar to PD patients. Each subject (including PD subjects) will complete a smell questionnaire and a sleep questionnaire to determine whether these “control” subjects have loss of smell or have REM sleep disorders because these

conditions increase the risk of PD. For assessing smell, we will use the UPSIT questionnaire [94, 95]. For assessing REM sleep disorder, we will use RBD1Q which consists of a single question, answered "yes" or "no," as follows: "Have you ever been told, or suspected yourself, that you seem to 'act out your dreams' while asleep (for example, punching, flailing your arms in the air, making running movements, etc.)?" [96] We predict that at least 20 PD patients/MSA will have both household spouse and first degree relative willing to provide stool samples for this study.

Second, in the longitudinal study, we will collect stool every 3 months (with 3 day diet questionnaire prior to each collection) over 12 months and determine microbiome composition over time. All PD subjects will undergo clinical assessments at baseline and end of the study to determine the severity of PD. These assessments will include: Movement Disorder Society-Sponsored Revision of the Unified Parkinson's Disease Rating Scale (MDS-UPDRS); Hoehn and Yahr Stage (HY); Non-Motor symptoms Scale (NMSS); Montreal Cognitive Assessment (MoCA). We will correlate the microbiome data with PD symptoms, diet and response to treatment and progression of disease. These studies will determine whether disease progression and factors such as PD medications and diet significantly impact microbiome composition. Further, we will determine whether changes in SCFA-producing bacteria (as part of Aim 1.1) and/or abnormal SCFA profiles (as part of Aim 1.3) correlate with severity of PD symptoms.

Inclusion/ Exclusion Criteria:

For Parkinson's disease subjects:
Inclusion criteria:
1. 40-80 years of age
2.Documented diagnosis of Parkinson's disease

- For cross sectional study- the Parkinson's disease stages 1-4 are eligible
- For longitudinal study- the Parkinson's disease stages 1-2.5 are eligible

Exclusion criteria:
1.History of GI diseases (except for hemorrhoids or occasional (<3 times a week) heartburn) like Inflammatory bowel disease or Celiac disease
2.Antibiotic use within last 12 weeks
3.Use of probiotic supplement except yogurt
4.Intentional change in diet
5. Chronic use of NSAIDS. A washout period of 3 weeks is needed before the subject could be enrolled into the study. Low dose aspirin is allowed

For household controls/ 1st degree relatives:
Inclusion criteria:
1.40-80 years of age
2.No clinical evidence of neurological disorders including Parkinson's disease
3.Live in the same household as the Parkinson Disease patient or is a first degree relative of the PD patient
Exclusion criteria:
1.History of GI diseases (except for hemorrhoids or occasional (<3 times a week) heartburn))like Inflammatory bowel disease or Celiac disease
2.Antibiotic use within last 12 weeks
3.Use of probiotic supplement except yogurt
4.Intentional change in diet
5. Chronic use of NSAIDS. A washout period of 3 weeks is needed before the subject could be enrolled into the study. Low dose aspirin is allowed

Aim 1.3. Determine how dysbiosis promotes PD pathogenesis by correlating microbiomes to metabolites
Rationale. Gut bacteria produce metabolites that impact neuronal function [6, 97]. Some of the most common microbial metabolites with profound physiologic effects are SCFAs, produced through fermentation of fibers in the lumen of the colon. Several studies have shown that the biological effect of SCFAs is dose dependent, as high butyrate is pro-inflammatory and toxic while low dose butyrate is anti-inflammatory [63]. Butyrate can also impact biological processes through epigenetic changes because it is a potent HDAC inhibitor [75]. SCFAs regulate intestinal barrier function and low levels, specifically butyrate and propionate, can promote gut permeability to bacterial products, leading to neuroinflammation [34], as has been seen in PD patients. Our data reveal feeding SCFAs to GF mice increases neuroinflammation (Sampson et al., *Cell*, 2016), consistent with a recent paper using the same approach [34], and also induces motor symptoms in germ-free ASO mice (see **Figure 3**). While robust, these findings are confounding in light of a report for low absolute SCFA levels in PD patients [33], though specific SCFAs are increased relative to total levels in PD samples. Additional research, as proposed in this project, is needed to reconcile these data by expanding PD cohorts for SCFA analysis, and experimentally overcoming potential developmental issues known for GF mice [98] by using humanized animals where bacteria dynamically produce SCFAs. We will determine ratios of SCFAs in PD patients, along with absolute levels. Finally, we will modulate SCFA ratios to test functional outcomes in mice (Aims 3 & 4).
Experimental Approach. We will determine SCFA concentrations in serum and stool from early onset PD patients and HCs. Briefly, as described in our studies [99, 100], samples will be thawed and centrifuged at 13,000 rpm for 10 minutes. An aliquot (0.2 μL) will be injected into a GC-FID 7890A instrument equipped with a fused silica capillary. Values will be quantitated by comparison to external standards for formate, acetate, butyrate, valerate, propionate, iso-butyrate and iso-valerate. This study will be the largest PD cohort to date that is analyzed for SCFAs, and the only one that will have metagenomic data. Thus, we will correlate serum and stool metabolomics data with stool metagenomic data, and PD symptoms and disease severity obtained through validated PD questionnaires. Principal component analysis (PCoA) of metagenomic and metabolomics data will be performed using MATLAB software (MathWorks) and compared to PD symptom data collected from subjects. Data will be centered and standardized ($\sigma = 1$) prior to running the PCoA function. We will use this bioinformatics analysis to define positive and negative correlations between all parameters, to determine a microbial or metabolite "fingerprint" for PD. In addition to informing the studies in Aims 2-4 and validating an involvement for SCFAs, this study may yield molecular biomarkers for early PD.

Expected Outcomes for Specific Aim 1. We anticipate confirming our prior findings that patients with PD have dysbiosis, and further identifying the nature of the dysbiosis including changes at the species and strain level. We will extend these studies via metagenomic analysis to quantify butyrate-producing bacteria and gene pathways involved in SCFA production, which we predict will be different between PD patients and HCs. We anticipate defining correlations between PD status, dysbiosis and SCFAs. We anticipate that microbiome composition in first-degree relatives is more similar to PD subjects than to HCs. We predict that patients with other forms of α-synucleinopathy have dysbiosis, but that their microbiomes are different from PD patients. Indeed, our pilot data using 16S rRNA analysis in 6 patients with MSA show distinct dysbiosis from PD (data not shown). We predict that patients with PD have abnormal SCFA ratios in stool and serum, and will carefully determine levels of all detectable SCFAs in order to determine disease-related profiles. We cannot predict microbiome stability in PD patients, but anticipate that there will be variation in the microbiome over time, but that dysbiosis will remain throughout the year with persistence in abnormal SCFA profiles.
Pitfalls and Alternative Strategies for Specific Aim 1. SCFA analysis and metagenomics are routine to our team. We do not predict any problems in collecting the proposed samples from subjects. We already have the required stool and serum samples from 40 PD patients and HCs. We have access to over 2,000 PD patients at RUMC where we have been obtaining samples for years, and several hundred more from the UW Movement Disorders clinics. We have extensive experience in collecting multiple stool samples over one year in PD patients with no dropout. Nonetheless, we plan to recruit an additional 4 subjects in the event of 10% dropout. We have access to patients with other synucleinopathies (over 70 patients with MSA and over 200 patients with Lewy body Dementia) through our Movement Disorders clinics at RUMC and UW. We have shown that the proposed number of subjects [n=40] is adequate for statistical differences in microbiome composition between PD and HCs [30]. However, if we find the number of samples in our biobank is not sufficient, we are able to collect additional samples at both sites (see letters from Drs. Shannon and Pal). If we find that our metabolomics data suggest non-SCFA pathways, then we will expand our studies to include comprehensive

untargeted metabolomics by sending samples to Metabolon (see letter of support), a commercial entity with whom we have collaborated and published [27, 90].

Specific aim 2. Determine how the microbiome regulates gut-brain interactions in a PD model

Introduction. Our preliminary data in **Figure 4** reveal that microbiota transplant from early onset PD patients exacerbates disease in ASO mice compared to gut bacteria from healthy controls. However, we did not measure SCFA levels in donors at time of collection, as sampling was prior to our current knowledge. Here, we will transplant microbiota into WT and ASO mice from high (top 3rd) and low (bottom 3rd) SCFA producers, aiming for 6 PD and 6 HC donors for each (24 total groups). We will assess motor symptoms and gut motility. Neuroinflammatory status will be determined, and αSyn aggregation measured in the gut and brain. We will extend our current data to measure neurodegeneration in the enteric nervous system and various brain regions using tissue clearing by CLARITY. CLARITY will be used to measure neuronal activation through cFos staining in fixed postmortem tissue; to corroborate and expand these results, we will perform real-time calcium imaging and electrophysiology in living tissue from humanized mice. These studies will reveal functional links between the microbiome and PD pathophysiology, and test SCFAs as a mediator of gut-brain connections.

Rationale. *Novel AAV tools to study cellular populations*. Genetically-encoded tools that can be used to visualize, monitor, and modulate mammalian neurons are revolutionizing neuroscience. The Gradinaru lab has recently published an *in vivo* selection platform (CREATE) for identifying adeno-associated viruses (AAVs) that more efficiently transduce genetically defined cell populations. The CREATE platform has already demonstrated one novel vector with intravascular delivery, AAV-PHP.B, that is 40- to 90-fold more efficient at transducing the brain than the current standard, AAV9 [101]. AAV-PHP.B transduces most neuronal types and glia across the brain. We also possess a novel, unpublished capsid (PHP.S) identified in the screen that has increased affinity for peripheral neurons over PHP.B or the widely used AAV9 (**Figure 5**).

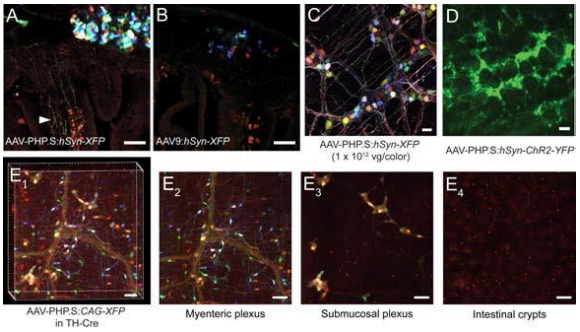


Figure 5. (A) Synapsin-driven multi-spectral labeling via systemically-delivered AAV-PHP.S appears brighter and labels fine processes (arrow) compared to **(B)** AAV9 in PACT-cleared duodenal cross sections of the mouse gut. **(C)** Individual neurons in enteric ganglia are distinctly colored, enabling tracing of individual neuronal processes. **(D)** Locally delivered synapsin-driven channelrhodopsin via AAV-PHP.S spatially restricts expression in enteric ganglia and limits photostimulation area. **(E1)** Systemic delivery of AAV-PHP.S coding CAG-driven Cre-dependent XFPs in TH-Cre mice restricts expression to dopaminergic neurons. **(E2-4)** Individual duodenal layers (18µm projections) are visualized in fine detail after PACT.

We will package constructs driven by cell specific promoters in AAV-PHP.S to achieve cell-type specific expression via non-invasive, systemic injection (by contrast, available vectors for local delivery can damage the gut). This will enable in depth observation (e.g. fluorescent proteins or calcium indicators) or manipulation (e.g. activity effectors) of peripheral nerves or enteric populations in the gut. Within these viruses, specific promoters will be used to drive the expected patterns of reporter expression in the CNS – we have had success with CaMKIIα for cortical glutamatergic neurons; Synapsin for neurons, broadly; GfABC1D for astrocytes; Myelin Basic Protein for oligodendrocytes; Tyrosine Hydroxylase (TH) for dopaminergic neurons; FEV for serotonergic neurons; and PCP2 for Purkinje neurons (**Figure 6**). We have also developed the VAST (Viral Assisted Spectral Tracing) system, which achieves sparse, multi-color labeling and will be used to perform tracing of dense neuronal pathways in both the gut and brain (**Figure 7**) and measure differences in neurites and synapses in experimental and control animal cohorts.

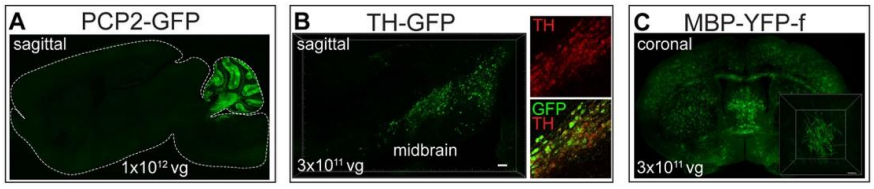


Figure 6. Intravenously delivered AAVs provide broad and cell type-restricted gene expression across the brain. A-C. Cell type-restricted promoters: PCP2, Purkinje neurons

(**A**); TH, midbrain dopaminergic neurons (**B**); and Myelin Basic Protein, oligodendrocytes (**C**) provide expected patterns of reporter expression within AAVs.

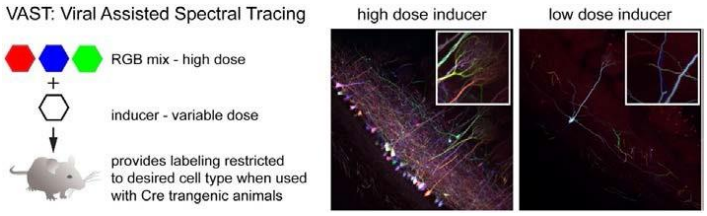


Figure 7. A modular AAV-based method for sparse, multi-color labeling with controllable labeling efficiency. The schematic shows the overview of the VAST approach, which takes advantage of the ability to deliver multiple transgenes to the CNS using bloodstream delivery of novel AAV serotypes such as AAV-PHP.B. VAST labeling of mitral cells and tufted cells in the mouse olfactory bulb in TBX-Cre mice at high (left) and low (right) doses of the inducer AAV.

Experimental Approach. *Behavioral analysis of colonized mice.* Prior to colonization, mice will be systemically injected with AAVs that contain promoter constructs to label Tyrosine Hydroxylase (TH) for dopaminergic neurons or Synapsin for all neurons. Wild-type (WT) or Thy1-ASO (ASO) mice raised in germ-free conditions will receive fecal transplants of high and low SCFA producing microbiota derived from PD and HC donors. After allowing 6 weeks of colonization, mice will be assessed for motor deficits and gut motility. After motor testing, mice will be sacrificed and tissues analyzed using CLARITY, as described below.

Motor function for all animals will be tested between hours 7 and 9 of the light-phase. Beam traversal is performed first, before allowing animals to rest for ~1hr and testing on pole descent. The following day, adhesive removal and hind limb scoring will performed. Fecal output will performed within 3 days and immediately prior to tissue collection. *Beam traversal*- A 1 meter plexiglass beam (Stark's Plastics, Forest Park, OH) was constructed of four segments of 0.25m in length. Each segment is of thinner widths 3.5cm, 2.5cm, 1.5cm, and 0.5cm, with 1cm overhangs placed 1cm below the surface of the beam. The widest segment is a loading platform for the animals and the narrowest end placed into home cage. Animals will have two days of training to traverse the length of the beam before testing. On the third day, animals will be timed over three trials to traverse from the loading platform to the home cage. Timing will begin when the animals place their fore limbs onto the 2.5cm segment and end when one fore limb reaches the home cage. *Pole descent*- A 0.5m long pole, 1cm in diameter, is placed into the home cage. Animals receive two days of training to descend from the top of the pole and into the home cage. On the test day, animals are placed head-down on the top of the pole and timed to descend back into the home cage. Timing begins when the experimenter releases the animal and ends when one hind limb reached the home cage base. *Adhesive removal*- ¼" round adhesive labels (Avery, Glendale, CA) will be placed on the nasal bridge between the nostrils and forehead. Animals are placed into their home cage (with cage mates removed) and timed to completely remove the sticker. Animals are recorded over three trials. *Hind limb clasping reflex scoring*- Animals are gently lifted upwards by the mid-section of the tail and observed over ~5-10 seconds [80]. Animals are assigned a score of 0, 1, 2, 3 based on the extent to which the hind limbs clasped inward. 0, no clasping. A score of 1 is assigned to animals which clasp one hind limb inward or if both legs exhibit partial inward clasping. A score of 2 is given if both legs clasp inward for the majority of the observation, but still exhibit some flexibility. A score of 3 is assigned if animals display complete paralysis of hind limbs that immediately clasped inward and exhibited no signs of flexibility. *Inverted grid*- Animals are placed in the center of a 30cm by 30cm screen with 1cm wide mesh. The screen is inverted head-over-tail and placed on supports ~40cm above an open cage with deep bedding. Animals are timed until they release their grip or remain for 60s. All motor tests are video recorded, and a blinded investigator scores performance to validate scoring by unblinded investigators. *Fecal Output*- Animals are removed from their home cages and placed into a 12cm x 25cm translucent cylinder. Fecal pellets are counted every 5 minutes, cumulative over 15 minutes. Principal component analysis (PCoA) of all motor function and gut motility is performed using MATLAB software (MathWorks) using behavioral data collected from mice that performed at least 3 tasks. Data is centered and standardized ($\sigma = 1$) prior to running the PCoA function.

After testing, mice will be sacrificed and prepared for either histology or physiological recordings. For these particular studies, we will colonize 12 mice per condition and all will undergo behavioral evaluation. Within each condition and as described below, tissue from 4 mice will be evaluated for histology, 4 for population activity imaging, and 4 for whole cell patch clamp electrophysiology.

Histological evaluation of neuronal populations in cleared brain and GI tissue. Mice will be sacrificed via intracardial perfusion with 4% paraformaldehyde. To enable fine-scale neural circuit mapping in a whole unsectioned specimen by light microscopy, the opaque sample needs to be "cleared" to allow light penetration

throughout the whole sample. One effective tissue clearing technology, CLARITY, creates tissue-hydrogel hybrids that lock in place bio-macromolecules and are amenable to selective lipid extractions to increase optical

transparency [102]. To perform this, GI and brain tissue will be collected then cleared using the passive CLARITY technique (PACT [103, 104]) developed by the Gradinaru lab by first embedding a 4% acrylamide hydrogel scaffold, hybridizing, and incubating in 8% SDS to remove light-scattering lipids. PACT clearing retains the three-dimensional structure of the tissue, preserves protein-protein interactions, and is compatible with fluorescent protein detection without the need for antibody labeling [105] (**Figure 8**). Antibodies will be used to stain tissue for various markers. In particular, we will use cell-specific markers to label neurons (PGP9.5), astrocytes (GFAP), microglia (Iba1), epithelial cells (E-cadherin), and neuron-specific markers to identify dopaminergic (TH) and cholinergic (ChAT) subpopulations. Antibodies against cFos will be used to detect neuronal activity. Antibodies to phosphorylated αSyn as well as a conformation-specific αSyn antibody will identify pathologic aggregates. See **Figure 2** as example of αSyn aggregation data. For microglia activation, IBA1 stained z-stacks will be imaged at 1µm steps and subsequently analyzed using Imaris software, as previously described [34]. Semi-automated reconstruction of microglia cell bodies and processes will be performed, whereby the experimenter designates individual cell bodies and the software quantifies diameter, dendrite length, and branch points from each given cell body. 20-60 cells per region per animal will be analyzed. PACT is also compatible with the TUNEL assay, and neurite labeling and quantification, which will be used to assess cell death/degeneration of TH-positive neurons. We will image this combinatorial labeling using confocal or light sheet microscopy to determine differences in cellular population activity, integrity, and death between conditions.

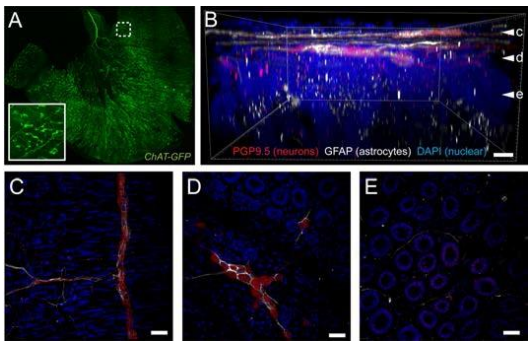


Figure 8. (A) Whole stomach from transgenic *ChAT-GFP* mouse cleared by PACT and imaged using light sheet microscopy. Inset shows retention of fine structure details of ganglia. **(B)** Antibody labeling of neurons (PGP9.5), astrocytes (GFAP), and nuclei (DAPI). Whole tissue was imaged using confocal microscopy and volumetrically reconstructed from 1µm thick images. Interactions between cell types can be clearly observed within and between **(C)** myenteric plexus, **(D)** submucosal plexus, and **(E)** intestinal crypts. Scale bars = 30µm.

Physiological recordings from brain and gut tissue. For recordings from the brain, thick slices (300µm) will be collected and incubated in oxygenated aCSF. Slices will then be mounted in a perfused chamber for recording. For recordings from GI tissue, 2 cm long segments of the GI tract will be cut and opened along the mesentery and incubated in oxygenated Kreb's solution with muscle relaxants (e.g. nicardipine, atropine). The tissue will then be microdissected to expose the myenteric or submucosal plexus and pinned to a perfused, Sylgard lined chamber, mucosa side up. We will perform whole cell patch clamp on both brain and GI tissue to assess passive (e.g. membrane resistance, resting potential), active (e.g. action potential threshold and amplitude), and synaptic (e.g. post synaptic currents) properties of neurons. In the brain, we will record from substantia nigra pars compacta. In the gut, we will record from both the myenteric and submucosal plexuses of the duodenum, jejunum, proximal colon, and distal colon. We will record a minimum of 12 neurons from each region in each mouse for comparison. By performing AAV-based labeling before microbiota transplantation as described above, specific cell types can easily be identified for recording. Separately, we will use AAVs to deliver a genetically encoded calcium indicator (e.g. GCaMP) for activity imaging. For the gut, tissue can be prepared without the need for microdissection and population dynamics can be recorded via fluorescent imaging in response to physiological stimuli (i.e. stretch, touch) [106].

Expected Outcomes for Specific Aim 2. We expect that ASO mice receiving transplants from PD donors will display increased pathology compared to mice with microbiota from HCs. Further, we predict animals receiving microbiota from low SCFA producers will display more severe motor deficits, while it may be difficult to resolve here which SCFAs mediate phenotypes (addressed in Aims 3 and 4). We expect to observe increased αSyn aggregation in both brain and gut tissue for animals with the worst motor defects. In the brain, we expect to see an increase in dopaminergic cell death or degeneration in mice transplanted with PD donor-derived microbiota, though phenotypes may be subtle such as deterioration of dopaminergic projections, primarily in the

striatum, while cell bodies are spared. Physiologically, we expect PD-microbiota colonized mice to display decreased enteric neuronal activity, particularly in the dopaminergic population [107]. We expect to observe reduced cellular excitability as well as decreases in postsynaptic currents generated by dopamine release.

Pitfalls and Alternative Strategies for Specific Aim 2. We expect no technical issues as all proposed studies are routine to the Gradinaru and Mazmanian laboratories, and all proposed technologies and reagents already generated and validated. Further, all IACUC, IRB and IBC protocols are already in place for the humanized mouse studies, as evidenced by our preliminary data. No studies have explored the physiology of enteric neurons in the context of PD models or during αSyn aggregation, thus data generated here will be novel. Preparation of gut tissue for whole cell patch clamp electrophysiology does risk damaging the enteric neuronal network during the dissection to expose the plexuses for recording. Using genetically encoded activity indicators circumvents this problem, however delivery of a high viral dose will be necessary to achieve strong expression in enough neuronal cells to observe population dynamics; we think this is possible based on pilot data for delivery of opsins at levels sufficient for photoactivation, using the AAV-PHP.S vector proposed here.

Specific aim 3. Develop and test "designer" fiber diets to modulate SCFA levels in PD mice

Introduction. SCFAs are normally produced in the colon as a consequence of fermentation of dietary fibers by subsets of intestinal bacteria, and impact the immune and nervous systems. Our data suggest that SCFAs contribute to the pathogenesis of PD. Indeed, feeding a mixture of SCFAs promotes motor symptoms in GF mice on an ASO background (see **Figure 3**). While butyrate activates microglia [108, 109], butyrate is anti-inflammatory in other contexts [109, 110]. One of the reasons for these contradictory results is our limited understanding of how SCFAs impact neuroinflammation and contribute to PD pathogenesis. Microglia may respond differently than other immune or epithelial cell types to SCFAs. Further, it is not known whether the absolute levels of each of the three main SCFAs, or rather the ratio of these SCFAs, are important and whether the rate of SCFA production influences their biological effects. We acknowledge that feeding defined mixtures of SCFAs to GF mice is an artificial approach, though it does provide a robust proof of concept. Further, dynamic bacterial production of SCFAs may provide different results than feeding static concentrations. Thus, in order to interrogate the impact of SCFAs in PD, it is most prudent to manipulate SCFA profiles (increases or decreases) using "authentic" approaches where gut microbes transform dietary fibers into SCFAs metabolites.

Aim 3.1. *In vitro* studies to identify designer fibers to normalize SCFA profile in PD stool

Rationale. Different fiber oligosaccharides and polysaccharides have distinct fermentation profiles favoring the growth of subsets of bacteria [111]. Thus, one can use this prebiotic property of fibers to modify gut microbiome composition and function to achieve "desired" biological outcomes. Of course, not all bacteria are capable of fermenting polysaccharides to produce SCFAs, and different bacteria produce different types of SCFAs. We have shown the importance of structure/function of fibers in producing specific SCFAs by gut bacteria, and demonstrated our ability to carefully change the structure of fiber products, leading to differential production of SCFAs when incubated with stool from PD patients and HCs (see **Figure 10**). Thus, changing SCFA profiles in stool with prebiotics is a strong tool to 1) elucidate the underlying mechanism of microbiota involvement in the pathogenesis of PD and 2) identify a therapeutic approach for PD.

We propose that studying the levels and/or ratios of SCFAs may shed light on their mechanism of action. We have developed a novel library of "designer" prebiotics that produce high or low butyrate and propionate proportions, and are slow fermenting compared to commercial fibers (**Figure 9**), thus avoiding production of rapid and high levels of SCFAs that may be toxic to cells. We further validated the specific SCFA-producing properties of our prebiotics in fermentation bioreactors by adding them to stools from PD patients and HCs, and showed that these prebiotics can shape the SCFA repertoire (**Figure 10**).

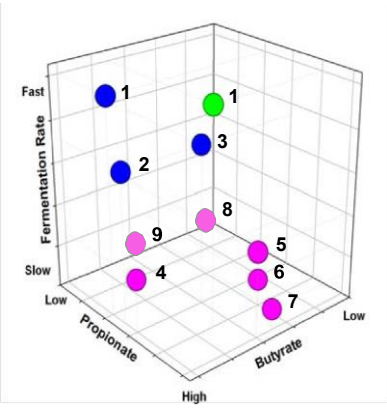


Figure 9. Conceptualized placement of dietary fibers that provide different relative proportions of butyrate and propionate, and further classified as slow or fast fermenting. Slow fermenting fibers [(in pink) 4=starch-entrapped microsphere, 5= soluble wheat bran arabinoxylan, 6 and 7=soluble corn bran arabinoxylans, 8=enzyme-debranched corn arabinoxylan, 9=resistant potato starch (all but #9 produced at Purdue)] are preferable to fast fermenting fibers [(in blue) commercial fibers, 1=fructooligosaccharides (FOS), 2=resistant high-amylose corn starch, 3=psyllium; and soluble sorghum bran arabinoxylans (prepared at Purdue) that was fast fermenting with high butyrate level]. Slow fermenting fibers will be chosen for animal testing due to their high tolerability and production of SCFAs into

more distal regions of the colon. In the current project, we will extend this analysis to acetate as well.

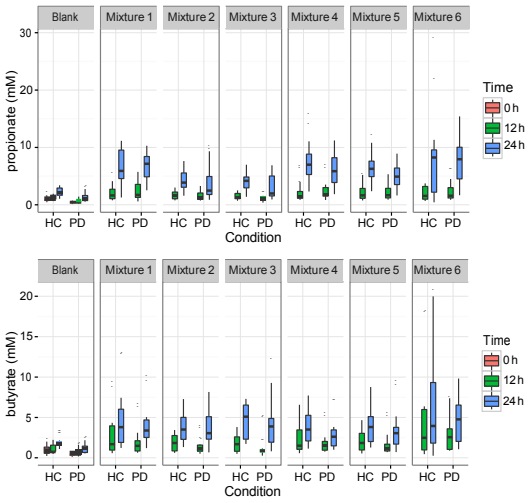


Figure 10. Prebiotic mixture diets tested in a fermentation bioreactor on healthy control (HC) and Parkinson's patient's (PD) stools. Certain diets were as propiogenic as the Mixture 6 fast-fermenting control (Mixtures 1 and 4), and as butyrogenic (Mixture 3). By 24 h fermentation time, most but not all of the fiber mixtures brought propionate and butyrate levels in PD stools to that of the HC group (PD initially had significantly lower propionate & butyrate). Mixtures were 1=20% FOS + 20% potato starch + 20% Promitor™ (soluble corn fiber) + 20% chitin-glucan + 20% wheat bran, 2=50% potato starch + 50% wheat bran, 3=50% potato starch + 50% chitin-glucan, 4=50% Promitor + 50% wheat bran, 5=33.3% potato starch + 33.3% Promitor + 33.3% wheat bran, 6 (soluble faster-fermenting control)=50% FOS + 50% Promitor.

In year 1, we will feed dietary fiber mixtures already shown in our *in vitro* bioreactor studies to produce "healthy" butyrate/propionate/acetate ratios in PD stools to mice, profile effects of these diets on the microbiome, and assess neuroinflammation, αSyn pathology, and motor symptoms (Aim 3.2; see below). These results will form the basis for studies in years 2 and 3 to further enhance the fiber-PD relationship with emphasis on identifying fiber chemical and physical structures that specifically enhance expansion of bacterial species that are propio- or butyro-genic for further testing in animals for PD-like symptoms. These studies will develop and test novel approaches for dietary control of microbial metabolites linked to PD.

Experimental approach. In year 1, we will test the three fiber products that are most effective to "normalize" SCFA profiles via increased butyrate/SCFA ratios, propionate/SCFA ratios and acetate/SCFA ratios (data for candidate fibers already collected; see **Figure 10** for partial list) in mouse models (see Aim 3.2). This "gain of function" approach should provide evidence for the importance of SCFAs and also to determine the potential importance of increased butyrate vs. propionate vs. acetate ratios in modifying disease progression in mice.

For the bioreactor studies in years 2 and 3, we will first test various fibers and fiber mixtures to understand better how to generate desired profiles of SCFAs for positive changes in PD endpoints, as determined in the year 1 study. Stools from 3 PD patients and 3 healthy controls, with divergent microbiota as determined by metagenomic sequencing, will be used in fermentation studies as described below. Then, 3 or more candidate fibers will be chosen and tested on stools collected from all 40 PD patients and 20 healthy controls in Aim 1.1. to study the effects of different fiber products on microbiota composition and SCFA production. Fibers will be studied that meet the pre-determined criteria of producing high SCFA amounts with relatively slow fermenting property for tolerability and distal fermentation in the colon. RT-qPCR with primers specific for the 16S rRNA gene will be used to track both butyrate (e.g., *Eubacterium*, *Rosburia*) and propionate producing bacteria (*Prevotella*, *Bacteroides*) groups, and followed by metagenomics for community profiling.

Fiber will be measured by the phenol sulfuric acid method and fibers adjusted to same fiber weights. Fecal samples provided by subjects will be, as quickly as possible, flash frozen in liquid nitrogen and thawed in the anaerobic chamber 30 minutes prior to analysis as per Rose et al. (2010) [112]. The bioreactor fermentation procedure will be similar to that described by Kaur et al. (2011) [99], except that all procedures will be done in an anaerobic chamber. Dietary fiber (1%) and 5% feces will be added to PBS buffer in an anaerobic tube, and sealed tubes incubated at 37 °C for 12 h, and in certain cases for a time course profile at various times from 0 to 24 h. Fermentation metabolites will be analyzed according to our protocol in Kaur et el. (2011) [99] by gas chromatography. Metagenomic analysis will be done as described in Aim 1.1. These studies will identify designer fibers that "normalize" SCFA ratios, or even improve upon SCFA ratios (i.e. elevate targeted SCFAs), in PD stool to that of healthy controls.

Aim 3.2. Normalizing SCFA ratios in a humanized mouse model of PD

Rationale. While bioreactors are useful due to high throughput capabilities, mouse models are needed to determine complex phenotypes. This sub-aim will test whether dietary management of the microbiome may be

a viable approach to modulate PD symptoms in humanized mice.

Experimental approach. We will generate independent mouse colonies harboring microbiota from 5 human PD patients, selected as those whose microbiomes and SCFA levels deviated the most from healthy controls, as

determined in Aim 1. 1. Both germ-free WT and ASO mice will be colonized. Beginning at 5-6 weeks of age, we will feed dietary fibers already known to increase ratios of the 3 major SCFAs continually in drinking water, and leave control groups unfed. Fiber doses will be determined empirically as the concentration in water required to achieve the desired output fiber concentrations in feces of GF mice (thus, accounting for background absorption and non-microbial breakdown). Starting at 12 weeks of age, mice will be motor tested, neuroinflammation determined and αSyn pathology assessed, as described in Aim 2. Microbiomes and metagenomes will be profiled, as described in Aim 1.1. SCFA levels will be tested in feces over time and colon contents upon sacrifice, to determine if metabolite profiles responded as predicted by the fiber administered.

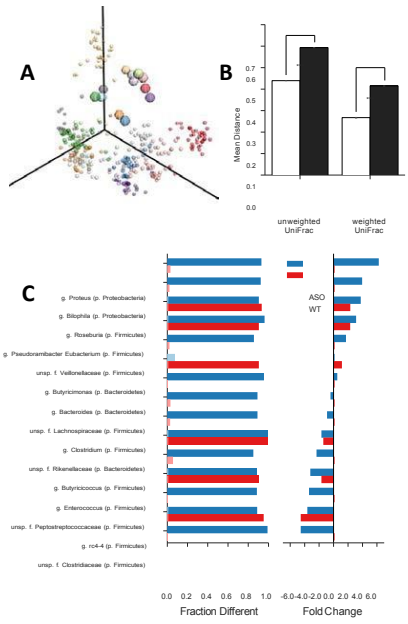
When available by years 2-3, "optimized" fibers from the bioreactor studies using PD and healthy stool from this project will be tested in humanized mice, as described above for both technical approach and outcome measures. Criteria will be identifying fibers that shift the microbiome and metabolome of the 5 most divergent PD donors toward a "healthy" state. The ability to develop fibers predicted to have effects for specific microbiomes, then test efficacy in mice with matched or different microbiomes, may also have implications in development of personalized mouse models of disease.

Expected outcomes for Specific Aim 3. We predict that we will find abnormal SCFA profiles in PD stool before fermentation and improvement of SCFA profile after fermentation with some of the fibers we plan to study. We predict that we will be able to identify at least 2 fiber products that show efficacy in mice following testing and identification from bioreactor studies. Finally, it would be exciting if a fiber had efficacy in the humanized mouse group that matched its bioreactor results, and reduced efficacy in other mouse groups.
Pitfalls and Alternative Strategies for Specific Aim 3. We do not predict any technical issues as the proposed methods are well established in our laboratories. If we find that the first 5 fiber mixtures we selected to study do not provide a desire outcome when co-cultured with PD stools, then we will use other sets of products that we have already characterized their fermentation profile and predict that they will be able to increase butyrate and propionate (see **Figure 9**) [100]. If we do not identify new products with better SCFA profiles, then we will continue to use the products that we have already identified to increase butyrate and propionate in PD stools (see **Figure 10**). Finally, as an alternative and possible future study, we will transplant into GF mice microbiota from PD patients before and after bioreactor "treatment" with fibers, and test motor performance and GI/brain pathophysiology to determine if reshaping the microbiome *in vitro* is sufficient to alter its function.

Specific aim 4. Test dietary and probiotic treatments in mice with PD-like symptoms
Introduction. We speculate that the efficiency of a microbiome to utilize dietary fibers varies based on its composition, and an optimal intervention requires modulating both diet and the microbiome. Further, different SCFA profiles likely impact gut bacterial composition. Herein, we will mine existing microbiome sequence data to identify bacteria that are selectively reduced or missing in PD patients, and treat humanized PD mice with novel probiotics to test for amelioration of motor symptoms and pathophysiology. Next, we will analyze microbiomes from the current project (Aim 1.1), which will expand our PD cohort from 6 to 40 subjects, to identify and test additional microbial treatments. Finally, we will feed specific diets that favorably shape SCFA ratios (as determined in Aim 3) while administering candidate therapeutic bacteria, to test the innovative hypothesis that both dietary and microbial interventions are needed for optimal benefits in a PD mouse model.

Aim 4.1. Administer to mice bacteria that are depleted in the PD microbiota and test symptoms
Rationale. Publications from our group and others have recently revealed that the intestinal microbiome is altered in PD patients relative to controls [30-32]. Further, we show that transplant of human gut bacteria into mice transfers symptoms from PD patients (see **Figure 4**), suggesting microbiome changes are functional and not simply a consequence of disease. In both the human studies and humanized mouse studies, it appears that specific microbes are selectively depleted in subjects with PD. Briefly, fecal pellets were collected from the "humanized" mouse groups whose data is shown in **Figure 4**, bacterial DNA extracted, and 16S rRNA sequencing performed in the laboratory of Dr. Knight. Sequences were annotated into Operational Taxonomic Units (OTUs). Recipient animal groups were most similar to their respective human donor's profile in unweighted UniFrac [113], based on PCoA (**Figures 11A, B**). We identified a number of genera that are altered in animals colonized with microbiota derived from PD donors, compared to healthy controls (**Figure 11C**). OTUs reduced or missing in ASO mice with PD microbiota include members of families Lachnospiraceae, Rikenellaceae, and Peptostreptococcaceae (**Figure 11C**). Intriguingly, we observe that animals receiving PD

donor-derived microbiota display an altered SCFA profile compared to mice colonized with microbes from controls (data not shown), supporting a link between dysbiosis and SCFA levels. Together, these results indicate



that differences in fecal microbial communities in PD patients can be maintained following transfer into mice, and suggest bacteria that are reduced in PD may be new probiotics.

Figure 11. Microbiome dysbiosis of PD patient samples after transplant into germ-free mice. (A) Unweighted UniFrac Principal Coordinate Analysis (PCoA) of microbial communities of human donors (large circles) and recipient mice (small circles). Each donor and recipient sample are matched by color. **(B)** Unweighted and weighted UniFrac analysis of microbial communities in recipient animals based on donor identity. **(C)** Taxa-level analysis of individual genera altered between PD and healthy donors as a function of recipient mouse genotype. Left column indicates percentage with significant differences observed; right column indicates fold change between PD and healthy donors. ***$p \leq 0.001$, 999 permutations. WT=wild-type, ASO=Thy1-α-synuclein genotype.

Experimental approach. We will treat humanized mice harboring a PD microbiota with bacteria that appear to correlate with health. Initially, we will leverage data gathered from our completed studies. Experimentally, we will colonize germ-free WT and ASO mice with microbiota from the 3 PD patients that displayed the most severe symptoms (Note: individual subjects will be chosen from the 6 PD patients for which the aggregate data is shown in **Figure 4**). GF mice will be colonized at 5 weeks of age to generate 3 independent mouse colonies. At 12 weeks of age, each animal group will be treated by oral gavage with 1) Lachnospiraceae; 2) Rikenellaceae; 3) Peptostreptococcaceae; or 4) mock gavage. Individual bacterial species will be chosen as those most phylogenticially similar to the OTUs from our data set and available from the American Type Culture Collection (ATCC; URL not provided as to adhere to DoD application guidelines). Bacteria will be grown in pure culture, and 1 x 10^9 colony forming units (CFU) will be administered to mice daily for 30 days; this regimen was chosen as it is a high dose and exposure, increasing likelihood for a positive result with future experiments aimed at optimizing treatment parameters. Fecal samples will be collected before and weekly during treatment for microbiome profiling. Following 30 days of treatment, mice will be tested for motor symptoms in beam traversal, pole decent, adhesive removal and hind limb clasping, as described in Specific aim 2, and shown in **Figure 3** and Sampson et al., 2016. For those microbial treatments that ameliorate motor deficits, mouse groups will be analyzed for neuroinflammation and αSyn aggregation as described in Aim 2. Finally, for those probiotic treatments that ameliorate motor symptoms, neuroinflammation and reduce αSyn aggregation, neuronal phenotyping and CLARITY will be performed comparing treated and untreated groups as described in Aim 2. This comprehensive analysis will not only identify novel microbial treatments for PD symptoms in mice, but is designed to reveal mechanisms of action for how beneficial bacteria may ameliorate the pathophysiologies that contribute to motor deficits.

In addition to the microbes to be tested from our existing data set, this project will generate new information that adds to the list of potentially therapeutic microbes based on a larger cohort size, as described in Aim 1. Depending on the number of new bacterial candidates, mice can be treated first with pools of species and then narrowed down, or treated with single species from the onset. Description of experimental approaches similar to the studies above. This research will identify additional microbes with therapeutic potential.

Aim 4.2. Combine prebiotic and probiotic treatments to optimize disease protection in mice

Rationale. A direct prediction from our central hypothesis is that a possible intervention for PD may require cooperation between diet and microbiome, since specific microbes metabolize certain dietary fibers into molecules that may impact PD. The studies proposed in Aim 3.2. will test potential therapeutic diets in mice with a PD microbiome (that may be missing key bacterial species), and Aim 4.1. will test candidate probiotics for efficacy without altering diet, which may be required for some microbes to improve PD symptoms. Herein, we will test if diets predicted to "normalize" the PD microbiome require specific species of bacteria for their efficacy in mouse models, suggesting that synbiotics (prebiotic + probiotic) may be the most optimal treatment.

Experimental Approach. We will generate independent mouse colonies harboring microbiota from 5 human PD patients, selected as those whose microbiomes and SCFA levels deviate the most from healthy controls, as

determined in Aim 1. Both germ-free WT and ASO mice will be colonized. The fibers to be tested will include all which showed effects on targeted SCFA modulation in bioreactor studies, regardless of efficacy in the mouse studies of Aim 3.2. The bacterial species to be tested will include those that are significantly depleted in the PD patients from this current cohort, as determined by Aim 1.1. Fibers will be tested individually by administration in water at doses empirically determined. Due to the numerous combinations of treatments and animal groups, mice will be treated with pools of candidate species (as consortia of bacteria may in fact be needed for efficacy), and then narrowed to single species in subsequent studies as needed. Also, we will not maintain all 5 donor-derived mouse colonies simultaneously, rather use economy of scale to test prebiotic and probiotics combinations in mice (WT and ASO) colonized from a single donor at a given point in time, as this will allow us to breed larger age- and sex-matched groups. All techniques used to profile the microbiome, determine SCFA levels, test motor and GI function, assess neuroinflammation, measure αSyn aggregation, and determine neuronal activity have already been described. These studies will test if diets predicted to "normalize" the PD microbiome require specific species of bacteria for their efficacy in mouse models.

Expected Outcomes for Specific Aim 4. We anticipate identifying bacteria (or collections of bacteria) that protect mice from PD symptoms, based on selective microbial depletion from the human PD population. Some species may protect without modulation of SCFA levels, while others may require specific dietary fibers for full efficacy. For those conditions where there is cooperation between diet and the microbiome, we predict that fibers producing "optimal" SCFA ratios will be most effective in ameliorating symptoms in mice.
Pitfalls and Alternative Strategies for Specific Aim 4. All techniques and reagents required for this project are already in use by our team, thus we anticipate no technical issues. While historically many gut bacteria were not culturable, recent advances now allow isolation of 60%-70% of the human microbiota [114, 115]. If the bacterial species of interest from the microbiome profiling are not closely represented phylogenetically by strains from the ATCC, we will culture and isolate desired species from human donor stool using selective microbiological plating and 16S rRNA matching to metagenomic data. For the bacterial treatment studies, dose and exposure schedules may need to be optimized for full efficacy. Finally, our analysis will identify microbes that are over-represented in the PD population, suggesting they may have pathogenic potential. If we fail to identify disease-protective microbes, we will administer possible PD-promoting bacteria to mice humanized with healthy microbiota, and measure motor symptoms and pathophysiology, as strategies to inhibit the activity of pathogenic microbes may be a therapeutic option. These studies can instead be performed in future projects.

Statistical analysis. Group descriptive statistics will be performed on all variables. Normality of distribution will be assessed by the Lilliefors test, and homogeneity of variance will be examined with the Levene test. Inter-group differences will be analyzed by one or two-way ANOVA using the Holm-Sidak test for comparison. For animal research, based on our previous studies over many years, 6-8 mice per group will be needed (and all studies performed in duplicate) on average in each group to achieve a probability of 88% that the study will detect a relationship between independent and dependent variables at a two-sided 5% significance level. This power calculation assumes that the true change in the dependent variables is 0.53 standard deviations per one standard deviation change in the independent variable. For human subjects work, based on the humanized mouse data in **Figure 4** and our publication [30], a sample size of 20 human donors per group achieves 96% power to detect a small effect size (f2= 0.08) attributed by patient category by an F-test with an alpha=0.05, when adjusting for 7 additional explanatory variables.

Overall Strategic Plan. De-identified stool samples collected at RUMC will be sent to Caltech for colonization of mice, as we have been doing for over 2 years. Additional samples for ongoing enrollment will be collected at RUMC and UW. Fecal swabs from all samples will be sent to UCSD for microbiome profiling (metagenomics). All samples will be coded so that investigators at Rush and UW can link it back to the subjects. Investigators from UCSD, Caltech and Purdue will not have access to the link to the codes with subject identifiers. Fibers will be developed and optimized in bioreactors at Purdue. Fecal samples and dietary fibers will be sent to Caltech, where all mouse treatments will take place. Fecal samples from Rush will also be sent to the Hamaker lab at Purdue to perform *in vitro* fermentation tests. The Mazmanian -laborator~~y~~ will administer the motor tests, assess neuroinflammation, and measure αSyn aggregation; while the Gradinaru laboratory will perform the CLARITY analysis and neuronal activity tests. Finally, fecal samples from mice will be sent to UCSD for metagenomics, and to Purdue for *in vitro* fermentation and SCFA analysis. We predict that specific, molecular interactions

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between diet and the microbiome impact PD outcomes, and that this project will identify mechanisms of action for how bacteria in the gut affect pathophysiology in the brains of mice. Merging the complementary expertise of the PIs and Co-Investigators, and using powerful preclinical models that bridge animal and human research, this project may define a functional contribution for the gut microbiome in a neurodegenerative disease that can be leveraged to develop novel, safe, effective and easy to administer treatments for Parkinson's disease.

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