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Madelyn C. Houser Date

Gene-Environment Interplay in Parkinson's Disease Pathogenesis: The Role of Gastrointestinal Inflammation and Regulator of G-protein Signaling 10 in the Gut-Brain Axis

By

Madelyn Crawford Houser
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Immunology and Molecular Pathogenesis

_____

Malú G. Tansey, PhD
Advisor

_____

John D. Altman, PhD
Committee Member

_____

Jennifer G. Mulle, MHS, PhD
Committee Member

_____

Andrew S. Neish, MD
Committee Member

_____

David S. Weiss, PhD
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____

Date

Gene-Environment Interplay in Parkinson's Disease Pathogenesis: The Role of Gastrointestinal Inflammation and Regulator of G-protein Signaling 10 in the Gut-Brain Axis

By

Madelyn Crawford Houser
B.S., University of Tennessee, 2013

Advisor: Malú G. Tansey

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ABSTRACT

Gene-Environment Interplay in Parkinson's Disease Pathogenesis: The Role of Gastrointestinal Inflammation and Regulator of G-protein Signaling 10 in the Gut-Brain Axis

By Madelyn C. Houser

The etiology of Parkinson's disease is unknown, but the prevalence of gastrointestinal problems in patients even before the onset of the characteristic motor impairments has prompted theories that pathology could develop initially in the gut and progress to the brain. The gastrointestinal abnormalities associated with Parkinson's disease could reflect the presence of chronic intestinal inflammation. If so, then inflammatory immune responses could link gastrointestinal symptoms with the neuroinflammation and neuropathology observed in Parkinson's disease.

In a cohort of human subjects, we confirmed greater incidence of intestinal disease and digestive problems in Parkinson's disease patients compared to controls. We sought to determine whether indications of intestinal inflammation could be detected in these patients but found that levels of immune-related factors in stool were strongly influenced by factors including sex, body mass index, smoking history, and probiotics use. When these variables were accounted for, we identified elevated levels of four proinflammatory molecules – IL-1α, CXCL8, IL-1β, and CRP – in stool from Parkinson's Disease patients.

To assess experimentally whether intestinal inflammation could exert pathological effects on the brain, we induced colitis in a mouse model and evaluated peripheral and neuroinflammation as well as neuron health in regions of the brain affected in Parkinson's disease. We also sought to determine how the effects of colitis might interact with sex, genetic predisposition to hyperinflammatory responses, and exposure to an additional neurotoxic insult. We discovered that in male mice, colitis promoted sustained inflammation and indications of $CD8^+$ T cell infiltration in the brain and perturbed dopaminergic neuronal activity without causing dopamine depletion. Colitis also augmented the impact of a known neurotoxicant in both sexes. These effects were particularly pronounced in mice that lacked the Regulator of G-Protein Signaling 10, which increased baseline intestinal inflammation and colitis severity.

This research confirms that intestinal inflammation is present in Parkinson's disease and that it has the potential to impact dopaminergic neurons in the brain and, when combined with other risk factors, to produce parkinsonian neuropathology. Controlling chronic gut inflammation could be an intervention that reduces the risk for the development of clinical manifestations of Parkinson's disease.

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CHAPTER 1: INTRODUCTION

Note: Portions of this Introduction have been previously published (1)

There is increasing awareness within the scientific and medical communities of the strong connection between the status of the intestinal environment and the function of the central nervous system (CNS). This so-called "gut-brain axis" incorporates bidirectional communication between the central and enteric nervous and endocrine systems as well as regulation of immune responses and inflammation in the gut and brain, and all aspects of this system appear to be heavily influenced by the activity of intestinal microbes (reviewed by (2),(3), and (4)). Much remains to be discovered regarding the content and consequences of the rich dialogue maintained between the CNS and the gastrointestinal (GI) system. Here, we focus on the potential for intestinal health to impact the brain and review evidence supporting the possibility that chronic intestinal inflammation may contribute to the development of neurodegenerative conditions such as Parkinson's disease (PD), and, as such, that particular regulators of intestinal inflammation may represent novel targets for study in PD.

1.1 Intestinal inflammation could promote the development of Parkinson's disease

1.1a Mechanisms of intestinal modulation of CNS activity

Numerous mechanisms mediate correspondence between the brain and the intestine. The most direct path is via the vagus nerve, which originates within the dorsal motor nucleus in the medulla and extends through the abdomen to the abdominal viscera. The vagus nerve provides the primary parasympathetic control of basic intestinal functions, with abundant innervation of the stomach, small intestine, and appendix that decreases proximal to distal, terminating before the distal colon (5). Stimuli in the intestine can trigger vagal afferent signaling, which is a critical

component of neuroimmune inflammatory reflex circuits that contribute to tonic peripheral immune regulation (6, 7). Emerging evidence consistent with the Braak hypothesis (8) described in detail below also suggests that the vagus nerve may act as a direct conduit by which material from the intestine can pass to the brain (9, 10).

Increasingly, the vibrant microbial community that occupies the intestine is also being identified as a key regulator of CNS activity. Changes in the composition of the intestinal bacterial population have been associated with a wide array of conditions including neurological and neurodevelopmental disorders such as multiple sclerosis (11), autism, depression, schizophrenia, and PD (12), and studies are beginning to explore some of the mechanisms that contribute to the powerful influence of the microbiota. Intestinal bacteria may exert direct effects on host processes through the production of signaling molecules that interact with the host nervous system, including hormones and neurotransmitters such as monoamines and GABA (13, 14). It has been shown that shifts in intestinal microbiota composition can alter the levels of some of these molecules along with levels of growth factors and signaling proteins in the brain (13), creating the potential for significant functional alterations. The microbiome also plays a significant role in controlling the release of a variety of gut peptides such as leptin and neuropeptide Y from enteroendocrine cells. Many of these molecules can act on the host nervous system and in fact play a key role in regulating circadian rhythms, anxiety levels, and behavior (14, 15). Gut bacteria are responsible for the conversion of primary bile acids produced by the liver to secondary bile acids, which are more readily absorbed through the intestinal epithelium. These bile acids can act as potent signaling molecules and regulate a variety of processes related to both the nervous and immune systems (16). Intestinal microbes are also the primary source of short-chain fatty acids (SCFAs). These molecules are known to significantly impact the gut

environment and host metabolism and to exhibit potent anti-oxidant and anti-inflammatory properties (2). In rats, SCFAs such as butyrate have been linked to increased colonic motility (17).The presence of SCFA-producing bacteria in the intestine has even been shown to strengthen the blood-brain barrier (BBB) by promoting increased expression and organization of BBB tight junction proteins (18). Metabolites from intestinal microbes such as those described here have also been reported to alter host gene expression in the brain, providing additional avenues for the microbiota to influence the activity of the CNS (reviewed by (13)). Interestingly, many, though not all, of the microbe-derived effects on the brain appear to be mediated through the vagus nerve (19).

The activities of intestinal microbes are inextricably linked to the status of the intestinal immune system. Under normal, healthy conditions, mucus and a tight barrier of epithelial cells confine most microbes to the intestinal lumen or the epithelial surface. Here, they stimulate homeostatic immune responses which predominantly promote tolerance of commensal microbes and the maintenance of barrier integrity (20) but do not cause significant inflammation, allowing microbes to persist in the intestine and execute their symbiotic functions (reviewed by(21)). The introduction of inflammatory triggers can upset this delicate relationship, however. Damage to the intestinal tissue, the introduction of aggressive pathogens, or exposure to substances that provoke strong immune reactions can increase the inflammatory quality of the intestinal environment. In turn, enteric inflammation can induce a number of effects that ultimately alter CNS function.

Immune cells can engage in direct communication with neurons (22). The extent of the functional impact of neuro-immune synapses is not known, but it is clear that activated immune cells can modulate neuronal activity via the release of neurotransmitters and cytokines (reviewed

by (23)). Local effects of inflammation on enteric neurons stimulate CNS responses via the vagus nerve (24, 25). Proinflammatory cytokines and activated immune cells in the circulation can also access the brain, particularly when the BBB is compromised, as it frequently is in aged individuals or in the context of neurodegenerative disease (26-29). Systemic inflammation can directly mediate BBB permeability. Extensive evidence has been reported linking molecules associated with inflammatory conditions including cytokines, reactive oxygen species, matrix metalloproteases, and mediators of angiogenesis with BBB disruption (30). Additionally, a positive feedback loop involving the traditionally proinflammatory cytokine interleukin 6 (IL-6) in conjunction with neuroimmune reflex circuits has been implicated in activating BBB "gateways" through which peripheral T cells gain access to the CNS and there contribute to neuroinflammation and neurodegeneration (28). Breaches in the BBB can significantly alter immune responses to CNS antigens (31) and compromise CNS protection against potentially harmful substances.

Perhaps the most well-characterized effects of intestinal inflammation on the CNS involve hyper-reactivity of the hypothalamic-pituitary-adrenal (HPA) axis and imbalances in serotonergic activity (reviewed by (23) and (32)). These changes have been associated with the manifestation of "sickness behavior" as well as anxiety and depression (33, 34), and these psychological conditions are frequently observed as comorbidities in individuals with diseases characterized by persistant intestinal inflammation, such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) (32, 35, 36). These and other systemic effects of intestinal inflammation are almost certainly mediated by a host of immune factors, but at present, the cytokines interleukin-1β (IL-1β), IL-6, and tumor necrosis factor (TNF) have been most frequently implicated (33, 34, 37, 38). Studies in rodents as well as clinical trials in humans have

demonstrated mitigation of these CNS changes induced by intestinal inflammation with specific inhibitors or antagonists of these cytokines (33, 39), with cyclooxygenase inhibitors (40), and with disruption of vagal signaling (37).

The systemic effects of intestinal inflammation may be further augmented by increases in intestinal permeability. Acute tissue injury may be incurred in a severe infection with an intestinal pathogen or in some forms of colitis, and this can cause temporary but substantial defects in the intestinal epithelial barrier. Low-grade inflammation normally induces more selective increases in paracellular permeability through regulation of tight junctions (reviewed by (41)). Intestinal microbes regulate expression of barrier-promoting tight junction proteins (20), and many proinflammatory cytokines secreted by activated immune cells – including TNF, IL-1β, and IL-6 – act on tight junctions to increase barrier permeability (42-44) in order to facilitate recruitment of additional immune cells and components from circulation to sites of inflammatory insult. Weakening of the intestinal barrier allows broader engagement of the immune system, but it also compromises the containment of gut contents, particularly microbial products, which can leak from the intestine into the peritoneal cavity and into the circulation, eliciting systemic proinflammatory immune responses (20). Typically, if the source of the immune challenge is rapidly cleared, proinflammatory responses terminate, and gut barrier function is restored. Unique features of the intestine, however, render it particularly susceptible to the development of persistent inflammation and barrier dysfunction.

With roughly 100 trillion bacteria in the intestine along with abundant fungi and viruses, the intestinal immune system is constantly exposed to microbial antigens which may serve as stimuli that prolong inflammatory responses. If host immune tolerance of the microbiota is sufficiently disrupted, chronic disorders characterized by abnormal intestinal inflammation and

permeability such as IBS or IBD may develop (45-47). Even if chronic disease does not manifest, low levels of intestinal inflammation such as those associated with obesity can significantly impact the microbiome, reducing diversity and altering bacterial population composition (48, 49). Inflammation seems to promote the selective survival of more aggressive microbes that have mechanisms for subverting or tolerating proinflammatory host immune responses – qualities characteristic of pathogens. Thus, under inflammatory conditions, the intestinal bacteria typically exhibit more pathogenic and less commensal activity, with increased mucosal invasion and reduced production of SCFAs and other beneficial metabolites (50-52). Translocation of microbes and reductions in levels of anti-inflammatory mediators further exacerbate inflammation and increase the likelihood of persistent immune responses in the intestine.

Sustained permeability of the intestinal barrier can have deleterious effects on numerous body systems. Many microbial components such as lipopolysaccharide (LPS) that enter the circulation with increased intestinal permeability are highly immunogenic and trigger systemic inflammatory responses. These responses, in turn, can promote further degradation of gut barrier function (53). The so-called “leaky gut syndrome” has been proposed as a contributing factor in a host of diseases. These include clearly GI-associated conditions like IBS, IBD, metabolic syndrome, and diabetes, but intestinal permeability is also being implicated in disorders of the CNS including autism, schizophrenia (54), multiple sclerosis (55), depression, anxiety, and post-traumatic stress disorder (56). Increases in systemic LPS can compromise both passive (57) and active (58) BBB mechanisms, rendering the CNS vulnerable to neurotoxic substances and activated immune cells from the periphery. Immune responses to circulating microbial antigens induce increases in proinflammatory cytokines in the periphery but also robust and persistent

increases in the brain (59, 60). This is likely facilitated at least in part by activation of microglia, CNS-resident immune cells (59-61). Proinflammatory responses in the brain can alter CNS function and behavior as previously described, and corrections in psychological and behavioral abnormalities accompanying resolution of inflammation and restoration of intestinal barrier function have been documented (62). CNS immune responses can have serious and enduring consequences, however, particularly if inflammation becomes chronic. Proinflammatory cytokines and oxidative stress have been causally linked to neuron death, and neuroinflammation is now considered a key factor in numerous neurodegenerative diseases (59, 63-71).

Many of these diseases are associated with advanced age, and as intestinal inflammation and forms of intestinal permeability have been documented to increase with age (72-74), immune mediation of gut-brain interaction may be particularly relevant in the pathology of neurodegenerative diseases of aging. One condition which has yielded some of the most substantial evidence of GI involvement is Parkinson's disease.

1.1b Clinical features of Parkinson's disease

PD represents a significant burden on our society, with over one million individuals presently affected in the U.S. and twice that number expected by 2040 (75, 76). It is more common in males than females, and it typically manifests in individuals aged 60 or older, with incidence increasing with age (77). PD is typically diagnosed in a neurologist's office on the basis of classic motor symptoms – most commonly a combination of bradykinesia, rigidity, resting tremor, and freezing (78) – that are caused primarily by the loss of striatal dopamine resulting from progressive degeneration and death of dopaminergic neurons in the midbrain. These symptoms are typically treated with dopamine-replacement therapy, but no currently-

available treatment slows the progression of PD-related neurodegeneration. Furthermore, by the time PD is diagnosed and treatment begins, extensive neurodegeneration has already occurred, as motor symptoms do not develop until 30-50% of nigrostriatal dopaminergic neurons and 60-70% of striatal dopamine have been lost (79). Therefore, the presence of clinical symptoms represents advanced stages of the disease, underscoring the need to identify and target early mechanisms of pathogenesis.

The precise causes of neurodegeneration in this disease have not been definitively established. Approximately 90% of cases are of idiopathic origin, and only 30% of inherited cases are attributable to known monogenic mutations (80). Abundant evidence has accumulated, however, demonstrating the presence of neuroinflammation in PD patients, and glial cell activation, proinflammatory signaling molecules, and oxidative stress are now considered by many to be key mechanisms that contribute to neurodegeneration in PD (68, 81-84). Another highly relevant factor in the pathogenesis of PD is the protein alpha-synuclein (αSYN). This molecule is present in numerous cell types throughout the body with high expression in presynaptic terminals of neurons, where it is thought to play a role in regulating vesicular release (85, 86). New studies continue to revise our understanding of αSYN's substantial conformational plasticity in normal physiology (87, 88), but it is clear that under certain circumstances, this protein adopts a β-sheet structure, loses membrane-binding capacity, and aggregates. This leads to the histological hallmark of PD –Lewy neurites and Lewy bodies composed of fibrillar, phosphorylated, ubiquitinated α-SYN (89-91), reviewed by (8)). These aggregations are found upon autopsy in the brains of individuals with PD, Lewy body dementia, and multiple system atrophy, and less reliably in other neurodegenerative disorders (92). It is, as yet, unclear whether Lewy bodies are themselves neurotoxic or whether they form as a protective response to

sequester toxic aggregated proteins and limit their potential to disrupt cellular organelles (93-98). The fact remains, however, that individuals with mutations in or over-expression of the αSYN gene *SNCA* usually develop early-onset, rapidly-progressing PD (80). The point mutations in *SNCA* associated with this phenotype stabilize β-sheet conformations, promoting aggregation with gain-of-function effects (99), and over-expression of αSYN has been shown to be sufficient to induce aggregation and neurodegeneration of dopaminergic neurons (reviewed by (97)) and is in fact the basis for several animal models of PD-like pathology. Given the abundance of evidence on the subject, it is quite likely that αSYN contributes either directly or indirectly to the pathogenesis of PD.

In addition to the well-established motor deficits, PD is also frequently characterized by an assortment of non-motor symptoms (NMS). One study determined that 98.6% of PD patients report at least one NMS, and on average eight NMS were identified per person (100). The most common of these symptoms include hyposmia, constipation, anxiety, rapid eye movement sleep behavior disorder, depression, excessive daytime sleepiness, impaired reaction time, and impaired executive function (101, 102). Some NMS in PD may be additional consequences of deficiencies in CNS dopaminergic activity or side effects of dopamine replacement therapy (103), but other NMS cannot be accounted for in this way, and as such may provide insight into underlying pathological mechanisms in PD. Furthermore, non-motor symptoms are often present in pre-clinical stages and have been observed with greater frequency in individuals who later develop PD compared to those who are not diagnosed with this condition (102, 104), suggesting that NMS may be manifestations of the earliest stages of PD, before dopaminergic neurons in the midbrain are affected. Recognizing pre-motor elements of this disease and defining mechanisms that regulate them may offer the potential for earlier diagnosis and more timely therapeutic

intervention that could delay or even prevent the development of the progressive motor symptoms of PD.

1.1c Intestinal involvement in PD

Recent evidence suggests that intestinal dysfunction is a non-motor symptom consistently associated with PD that may precede motor symptoms by decades. Constipation is the most common GI complaint (105) and the second most common NMS behind hyposmia in PD (102). Studies report constipation in 20-80% of PD patients (106, 107), and a meta-analysis places the incidence at 50% (102). Importantly, though, when colonic transit time in PD is measured objectively rather than determined through self-reporting on questionnaires, constipation prevalence is found to be higher, with nearly 80% of patients affected (108). Intestinal motility is largely controlled by the enteric nervous system (ENS) (reviewed by (109), but there is presently no consensus on whether PD-associated constipation occurs as a result of neurodegeneration within the ENS, the CNS, both, or as a consequence of another process entirely (52, 110-115). It is clear, however, that constipation can manifest as a pre-motor symptom years before CNS degeneration prompts a diagnosis of PD. One study reported that middle-aged men who had less than one bowel movement per day had over four-fold increased risk for PD diagnosis over the next 24 years compared to men with regular bowel movements (116). Another study found that constipated men (three or fewer bowel movements per week) were five times more likely and constipated women three times more likely to be diagnosed with PD within six years compared to individuals who were not constipated (117). Meta-analysis suggests that constipation is more than twice as common in people who develop PD compared to those who do not (102), and that constipated individuals are twice as likely to develop PD within 10 years of their evaluation

(118). The duration of time over which constipation is predictive of PD development is remarkable, but both prospective and retrospective studies have found that constipation becomes apparent an average of 15.6-24 years before PD is diagnosed (107, 116, 119, 120), making it one of the earliest indicators of pathological processes that ultimately lead to PD.

Another intestinal feature of PD that has been widely reported is the presence of enteric abnormalities in αSYN. This protein is expressed as a normal component of the enteric nervous system, and it can be detected in intestinal tissue in a large percentage of neurologically-intact humans (121-126). Numerous studies indicate, however, that αSYN is detected more frequently and at higher levels in the intestines of PD patients than in age-matched healthy controls (121, 127-131). This is significant, as over-expression of αSYN is known to produce αSYN aggregation in both the intestines and brains of mice and humans (80, 132, 133). Instances of phosphorylated and aggregated αSYN have been observed in the esophagus, stomach, small intestine, colon, and rectum of PD patients, but it has been suggested that they occur in a proximal to distal gradient, with the lowest incidence in the rectum (126, 134, 135). Most studies that have evaluated enteric synucleinopathy in PD patients have reported detection of phosphorylated αSYN in 61.5-80% of PD samples and Lewy bodies in 72.4-100% of PD samples, with 0-33% of healthy controls positive (135-139). This would suggest that intestinal synucleinopathy is a relatively sensitive and specific indicator of PD pathology. Furthermore, additional studies have reported distinctive αSYN immunoreactivity in intestinal biopsies taken from clinically normal individuals who would later develop PD (127-129), indicating that abnormal enteric αSYN is present before CNS neurodegeneration has advanced sufficiently to produce motor symptoms.

These findings regarding the distinctive features of intestinal αSYN in PD are not universally observed, however. A few studies have reported detection of phosphorylated synuclein in nearly all biopsies tested, regardless of PD disease status, or have otherwise found no differences between PD patients and controls (125, 140, 141). While differences in exclusion criteria among studies may account for some of these discrepancies (e.g. if all neurologically unimpaired subjects have colon cancer (124)), a key reason is likely differences in methodology. It has been shown that the detection of various forms of αSYN can change dramatically depending on the tissue preparation and the methodology employed (140-143). In fact, one study employed seven different immunohistochemical methods with five different antibodies on the same set of samples and determined that only one method accurately distinguished PD patients from controls (144). This study also emphasized the importance of utilizing full-thickness colon sections rather than biopsies to ensure that submucosal neuronal tissue was consistently present and of establishing a clear definition for positive staining that required recognizable neuronal morphology (144). It also revealed a significant degree of inconsistency in the interpretation of results by different blinded researchers (144), which renders the optimization and standardization of procedures for this kind of experiment even more essential before, as has been suggested, enteric synucleinopathy could be considered for use as an early risk indicator for PD.

Some researchers argue that phosphorylated αSYN and Lewy bodies in the intestine are not specific features of PD as they have been identified in the intestine of patients with other disorders such as achalasia (145), Lewy body dementia, incidental Lewy body disease, and Alzheimer's with Lewy bodies (135). It is worth noting, however, that these disorders share many pathological features with PD, and it has been proposed that at least some of these conditions may actually represent early, pre-motor stages of PD (8, 146-148). This concept may

be supported by observations that the extent of intestinal synucleinopathy in these conditions is not as advanced as that observed in PD patients (135). Furthermore, a recent study which did detect αSyn and phosphorylated αSyn in subjects with no known neurological impairment observed that individuals with positive αSYN immunostaining exhibited more of the non-motor symptoms and risk factors associated with PD than did individuals who showed no colonic αSYN (124). It is possible, then, that efforts to identify enteric αSYN manifestations which are specific to PD by comparing PD patients with subjects who have not been diagnosed with PD may be complicated by the possibility that undiagnosed individuals could be experiencing prodromal manifestations of PD or health issues which also elicit changes in αSYN in the gut.

This would be even more likely, if, as other studies have suggested, synucleinopathy may be a normal occurrence associated with aging. In 2012, Bottner *et al.* reported that phosphorylated αSYN could be found consistently in colon tissue from aged individuals (123). This was supported by a study in healthy rats which found increasing numbers of αSYN^{+} neurons and increasing αSYN aggregation in the colon as animals aged (149). A human study published in the same year, however, found no significant differences in αSYN expression levels in individuals from ages 40 to 91 (121). Regardless of whether or not αSYN expression increases with age, as αSYN has great conformational plasticity, and as clearance mechanisms for misfolded and aggregated proteins tend to deteriorate with age (150-152), it would not be surprising if αSYN aggregates were more commonly found in the intestine and throughout the body with increasing age. Individuals who develop PD may have greater impairment of clearance mechanisms or other predisposing factors that could contribute to propelling them over the threshold from normal aging into disease.

Clearly, questions remain regarding the prevalence, specificity, dynamics, and functional relevance of αSYN pathology in the intestine. Particularly if PD represents an extreme on a spectrum of αSYN abnormalities, then levels of expression, degree of post-translational modification, proportions of affected cells, and abundance of aggregates may provide greater insight into the role of this protein in disease pathogenesis than do the binary presence or absence data currently reported in most of the published literature. Only when consistent methods of tissue collection, preparation, assay, analysis, and interpretation have been established can we begin to address important questions regarding the function of gastrointestinal αSYN in the development and progression of PD and how it might relate to non-motor symptoms of the disorder.

Another intestinal component of PD that is receiving increasing attention is alterations in barrier function. A number of reports now indicate that PD patients have increased intestinal permeability compared to healthy controls (56, 131, 139, 153, 154). The results obtained from several of these studies specifically indicate defects in intestinal tight junctions without gross mucosal damage, including reductions in levels of barrier-promoting proteins and disruptions of tight junction networks (139, 153, 154), a phenotype consistent with low-grade inflammation (41). Defects may also be present in other intestinal barriers that limit direct microbial interaction with tissue, as instances of *Escherichia coli* penetration into the intestinal mucosa were more frequent in PD patients than controls and correlated, as might be expected, with increases in intestinal permeability and with oxidative stress, a component of inflammation (131). Interestingly, levels of enteric αSYN were also found to correlate positively with gut permeability and oxidative stress (131). To our knowledge, no reports have yet been published that assess intestinal barrier function in asymptomatic individuals who later develop PD, so the

extent to which increased permeability may be a pre-motor symptom of PD has not been established. Increased intestinal permeability was detected in newly diagnosed PD patients, though, so it can be concluded that it is present at least from the earliest clinical stages of the disease (131).

The relationships between gastrointestinal microbes and PD pathology have increasingly become subjects of active investigation. For years, the involvement of species of *Helicobacter*, a bacterium that colonizes the stomach and can cause gastric ulceration and ultimately gastric cancer, in PD has been proposed. In 1999, Charlett *et al.* reported that PD patients as well as their siblings exhibit greater frequency of *Helicobacter pylori* (*H. pylori*) seropositivity (155), and a 2013 study reported greater incidence of *H. suis* in gastric biopsies from idiopathic PD patients compared to healthy controls (156). Another study in the same year, however, found no differences in frequencies of *H. pylori* infection in PD patients and controls (157). Most studies report *Helicobacter* infection in roughly a third of PD patients (156-158), and a meta-analysis found that *Helicobacter* infection frequency in PD did not differ significantly from the general population (159). Though these discrepancies diminish the likelihood that *Helicobacter* infection is a significant risk factor for the development of PD, one study did argue to the contrary, reporting a 23-45% enhanced risk for PD development. The results of this study are difficult to interpret, however, as the authors did not examine confirmed *Helicobacter* infection as a risk factor, but rather the prescription of drugs used to treat *Helicobacter* infection as least five years prior to a PD diagnosis (160).

Perhaps the most robust findings connecting *Helicobacter spp.* with PD are in regard to improvement of motor symptoms. Several studies report that PD patients infected with *Helicobacter* exhibit greater motor impairment compared to uninfected patients (158), and that

treatment of the infection improves motor function (161). It has been established that *Helicobacter* can limit the bioavailability of levodopa (162), one of the most common PD treatments, so it is possible that eradication may improve motor symptoms simply by increasing treatment efficacy. However, two related studies reported improvements in stride length in PD patients following successful elimination of *Helicobacter* regardless of whether or not the patients were taking anti-parkinsonian medication (163, 164). Thus, there is evidence to suggest that *Helicobacter* infection may exacerbate PD pathology. A number of researchers have proposed that this effect may be due to chronic alteration of immune activity in individuals infected with *H. pylori* (160, 165-167), and as has been discussed, inflammation is a highly relevant factor in the development and progression of PD pathology.

Another microbial factor that is being evaluated with regard to PD is the abundance of gut bacteria. A greater incidence of small intestinal bacterial overgrowth (SIBO) consisting of increased bacterial density and dysbiosis in the small bowel has been reported in PD patients compared to unaffected controls (157, 168-170). The prevalence of SIBO in PD patients in these studies ranged from 25-54.5% compared to 8.33-20% in unaffected controls. One study reports greater frequency of SIBO in recently-diagnosed PD patients (169), while another found increased frequency with advanced disease (168), so there is no consensus on when this symptom develops in the course of the disease. As SIBO can result from diminished intestinal motility (171), it may be that this condition emerges when PD-associated neuropathology in the intestine has advanced sufficiently to increase small intestine transit time. Like *Helicobacter* infection, the presence of SIBO has been associated with greater PD-related motor impairment (169) and with fluctuating responses to levodopa, and antibiotic treatment has been shown to temporarily improve these symptoms (157).

Only recently have more detailed assessments of the intestinal microbiota in PD been initiated. Sixteen published studies have now characterized the composition of fecal or intestinal bacteria populations in different cohorts of PD patients and healthy controls, and some have also evaluated metagenomics and measured concentrations of microbial metabolites (52, 153, 172-185). When comparing the microbiota in feces or colon biopsies, more differences were found between PD patients and controls in fecal rather than tissue-adherent bacteria (183). Numerous different data acquisition methodologies and analyses were employed by the different studies, and while all have reported significant differences in microbiota composition between PD patients and healthy controls, there has not been broad agreement regarding the particular taxa that differ (reviewed by (186)). Nonetheless, several meaningful patterns are beginning to emerge.

Numerous studies have reported decreases in the relative abundance of bacteria that produce SCFAs such as butyrate as well as other beneficial, anti-inflammatory metabolites (52, 91, 173, 174, 178, 180, 182, 183). These include reductions in *Prevotella* and *Clostridium spp*. One study confirmed the functional impact of these changes, detecting reduced concentrations of fecal SCFAs in PD patients compared to healthy controls (184). The relative abundances of hydrogen-producing taxa are also reduced in PD (153), and molecular hydrogen functions as an antioxidant (187). Furthermore, *Prevotella* and *Clostridiales* are key producers of folate (52, 188) and thiamine (52) as well as SCFAs. Butyrate, hydrogen, folate, and thiamine have all been independently associated with amelioration of PD pathology (189-192). These modulations in the microbiome and its byproducts could contribute to impairments in GI motility and barrier function and to an inflammatory environment in the gut.

Metagenomics analyses and pathway prediction based on 16S sequencing data have revealed significant reductions in genes associated with normal metabolic activities (178, 180, 183) and significant increases in resources devoted to synthesis of LPS, type III bacterial secretion systems, (183) and efflux pumps (172), which are often involved in pathogenic interactions with host cells. In short, the fecal microbiota composition in PD patients appears deficient in microbes that mediate mutualistic anti-inflammatory and metabolic activities and enriched in pathobionts that stimulate inflammation and more readily induce damage to host tissue. One study also reported enrichment of genes associated with degradation of xenobiotics including herbicides and pesticides in the microbiome of PD patients (178). This is in keeping with studies identifying exposure to herbicides and pesticides as risk factors for the development of PD (193-195).

Finally, assessments of the microbiome in PD have identified correlations between the changes in relative abundance of particular taxa and the clinical symptoms of PD (reviewed by (186)). The first study to do so reported an association between increased *Enterobacteriaceae* in the feces of PD patients and postural instability and gait difficulty (52). Though members of the *Enterobacteriaceae* family can be components of the normal microbiota, this taxon has also been clearly linked to inflammation and disease. It includes potent inducers of immune responses, and *Enterobacteriaceae* levels in the intestine correlate strongly with indicators of inflammation (196, 197). Bacteria in this family can exhibit pathogenic qualities when normal immune constraints are lifted, as with the invasive *E. coli* observed in the colonic mucosa of PD patients (131). The relative abundance of *Enterobacteriaceae* surges when microbial diversity is reduced, as it often is in inflammatory or dysbiotic conditions. Abnormally high levels of *Enterobacteriaceae* are found in the intestine in humans and mice with inflammatory bowel

disease, colorectal cancer (198), dextran sodium sulfate-induced colitis (197), obesity (199), liver cirrhosis (200), and others. Correspondingly, levels of SCFA-producing taxa are often reduced under conditions of inflammation and illness (201-203), and reductions in these taxa in PD patients have been correlated with more severe motor symptoms including higher frequencies of postural instability and gait difficulty as well as cognitive impairment (174, 177). Sampson *et al*. confirmed the causative nature of these associations by demonstrating that germ-free αSYN-overexpressing mice colonized with fecal bacteria from PD patients developed more severe physical impairment than mice colonized with fecal bacteria from healthy human donors (204). The observed changes in the microbiome of PD patients support the concept that intestinal inflammation occurs in PD and that its effects on the gut environment are associated with neurological impairment in the CNS.

At this time, it is not clear whether the observed changes in microbiota in PD patients are an initial occurrence that contributes to the development of neurological dysfunction and degeneration, or if they emerge in response to PD-related pathology in the enteric and/or the central nervous systems. One exciting recent study has reported, however, that the composition of the microbiota in individuals with random eye movement behavior sleep disorder, commonly considered to be a manifestation of prodromal PD, differs from healthy controls and resembles that of PD patients, suggesting that dysbiotic alterations develop before the onset of motor symptoms (177). Regardless, because intestinal microbes can impact many PD symptoms, they represent a potential target for intervention that could at least help mitigate symptom severity and at best, inhibit PD pathogenesis.

1.1d Model of gut-originating, inflammation-driven PD pathogenesis

We have described how CNS function is influenced by intestinal microbes and the molecules that they produce which together act to stimulate enteric immune activity and regulate gut permeability. Sustained inflammatory conditions in the intestine can promote systemic inflammation and neuroinflammation. PD is characterized by intestinal dysfunction that can start over two decades before the onset of motor symptoms, and the observed constipation, intestinal permeability, dysbiosis, and increased levels of potentially pathogenic forms of enteric αSYN are all consistent with conditions of GI inflammation (41, 49, 205, 206). In fact, a few studies have reported observing signs of inflammation and oxidative stress in the gut of PD patients (130, 131, 173, 207, 208). On the basis of this accumulated information, the following model of PD pathogenesis can be formulated (**Fig 1**).

An initial inflammatory trigger is introduced. This could be a toxic substance such as the pesticides or pollutants that have been associated with PD (194, 209, 210) that induces damage and subsequent inflammation in the intestine. Many have proposed that an infection which directly or indirectly affects the GI system could be the initiating factor (147, 211-214); or perhaps it is more accurately the accumulated inflammatory burden of multiple infections, which has been linked to PD. Irritable bowel syndrome or inflammatory bowel disease, which are associated with low-grade and more severe GI inflammation, respectively, have been reported to increase risk for PD development (215-218), and these would certainly create inflammatory conditions which, for many individuals, may be chronic.

Sustained, low-level inflammation develops. If the immune responses elicited by the trigger are not resolved promptly, they would be expected to contribute to deleterious shifts in microbiota composition and to intestinal permeability which would allow leakage of microbial products and inflammatory mediators from the intestine. These could prompt systemic immune

responses which, among other consequences, could induce permeability of the BBB. Systemic inflammation has, in fact, been commonly reported in PD patients (219-224), as has BBB dysfunction (29, 225).

Synucleinopathy develops and exacerbates inflammation. Proinflammatory immune activity and conditions that elicit it have been shown to increase levels of αSYN in the gut and the brain (206, 226, 227). αSyn over-expression can then induce aggregation (reviewed by (97). Another factor that may contribute to the development of synucleinopathy in the intestine is the microbiota. Several studies have demonstrated that bacterial products including amyloids and even LPS can serve as nucleating factors for αSYN, promoting its aggregation (228-230). Over-expressed and aggregated αSYN would in turn stimulate proinflammatory responses from immune cells (231-237), initiating a positive feedback loop that could promote the spread of aggregated αSYN (Reviewed by (238)).

αSYN pathology in the periphery can transfer to the brain. While prolonged systemic inflammation resulting from intestinal pathology may on its own be sufficient to pathologically modify αSYN in the CNS, peripheral inflammation may also increase uptake of αSYN from circulation into the CNS by promoting disruption of the BBB (239). Furthermore, it has been demonstrated that human αSYN introduced into the intestinal wall of rats could migrate up the vagus nerve to the dorsal motor nucleus of the vagus (DMV) in the brainstem (10). This translocation was mediated by microtubule-associated transport in neurons, and it was observed equally for monomeric, oligomeric, and fibrillar forms of αSYN (10). Other studies have also demonstrated that αSYN can propagate through intact neurons (240) including throughout the ENS and to the CNS (241). These proof-of-concept studies suggest that changes induced in αSYN in the gut can directly affect the brain. In the brain, αSYN activates microglia (237) which

may already be primed by ongoing GI and systemic immune responses. It has been shown that peripheral inflammation exacerbates inflammatory responses to αSYN in the CNS (234), increasing the likelihood and accelerating the timeline in which neuroinflammation produces neurodegeneration.

CNS pathology in PD begins in the DMV. Numerous reports indicate that the DMV is consistently affected in PD patients (reviewed by (109)), exhibiting αSYN inclusions (242) and neurodegeneration (243). Heiko Braak and colleagues proposed a staging system for the progression of PD pathology through the brain with involvement of the DMV as the first stage (8). While questions have been raised regarding Braak's staging scheme and the methodology of his study (244-247), numerous publications have provided support for much of Braak's staging. Even studies designed to refute his proposal have still found that a majority of PD cases (53-81.7%) adhere to it fully, and only a small fraction (7-8.3%) do not show pathology in the DMV when it is present elsewhere in the brain (245, 246). Thus, it would appear that the majority of PD patients exhibit a form of the disease that follows a specific pathological progression in which the DMV is prominently affected. It is possible that the deviations from standard findings could result from differences in disease manifestations between, for instance, inherited, possibly monogenic forms of PD and sporadic cases or cases related to brain injury, as few studies report these categorizations.

PD pathology spreads throughout the brain. From the DMV, synucleinopathy, inflammation, and neuronal dysfunction would then propagate to other brain regions, ultimately reaching the substantia nigra, where dopaminergic neurons, which are particularly sensitive to inflammation (248), begin to degenerate. When sufficient depletion of striatal dopamine

resulting from loss of these neurons has occurred, motor impairments begin to manifest, and clinical symptoms of PD have developed.

This is only one model of PD pathogenesis that may apply in only a subset of patients, but it has robust support in the literature, and its elements are becoming increasingly common proposals in the field of PD research. In addition to the collection of intestinal symptoms long associated with PD that suggest a gut origin for the disease, it has been reported that in a mouse model with over-expression of mutant human αSYN, enteric abnormalities appeared before any CNS pathology (249). Other studies have shown that when rotenone, a pesticide commonly used to induce parkinsonian pathology in rodents, was delivered into the stomach of mice, it was found to induce enteric neurons to release αSYN, which then propagated to other neurons by retrograde axonal transport (250), accompanied by local inflammation (251). αSYN accumulation and phosphorylation appeared sequentially in the ENS, the DMV, and then other brain regions in accordance with Braak's proposed PD staging (251). This progression could be halted by resection of autonomic nerves (250). Accordingly, severance of the vagus nerve was recently found to be associated with reduced risk of PD in humans (252). The inflammatory aspects of PD are well established, and the concept of immune activation originating in the gut can link numerous risk factors, pre-motor, motor, and motor symptoms into a cohesive hypothesis of PD pathogenesis that can explain much of the observed disease presentation.

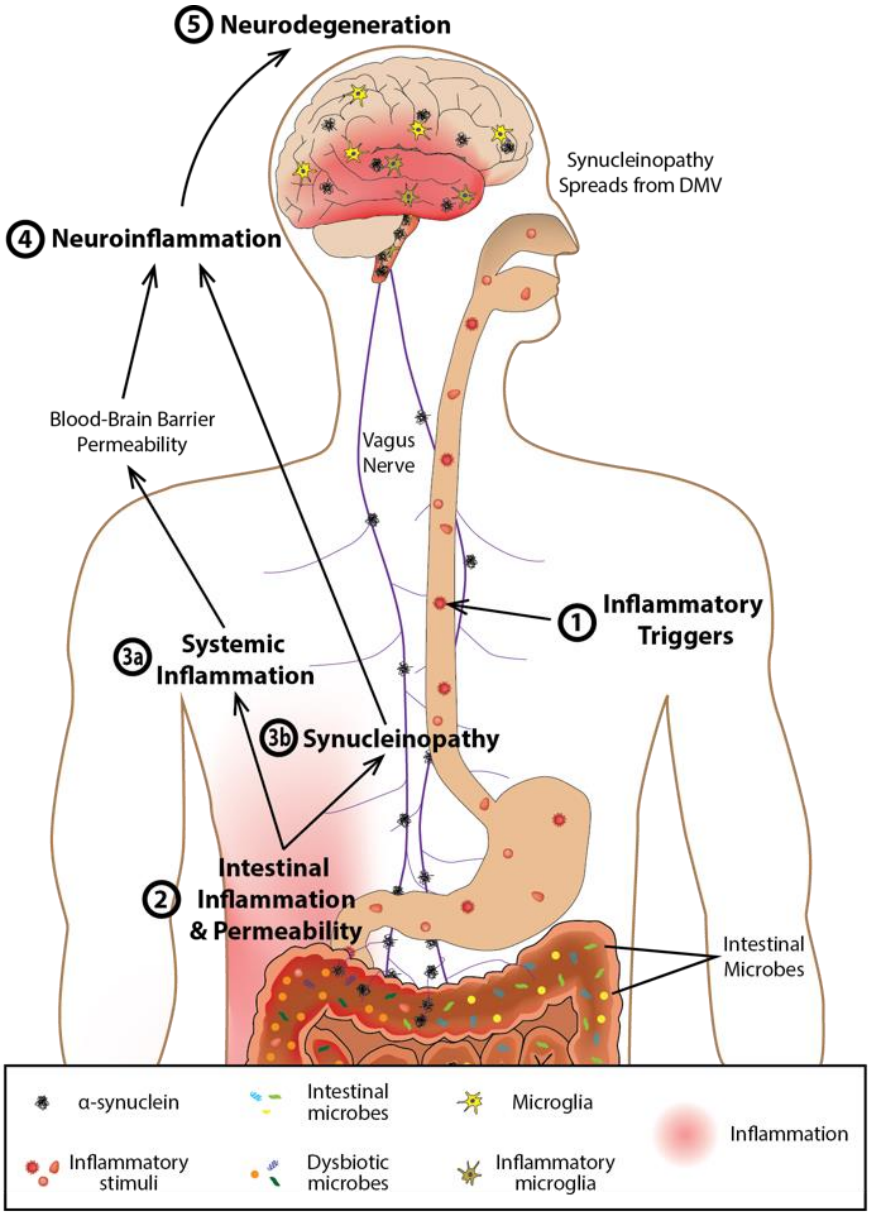

Figure 1: Model of gut-originating, inflammation-driven PD pathogenesis

In a susceptible individual, inflammatory triggers (1) initiate immune responses in the gut that deleteriously impact the microbiota, increase intestinal permeability, and induce increased expression and aggregation of αSYN (2). Synucleinopathy may be transmitted from the gut to the brain via the vagus nerve (3b), and chronic intestinal inflammation and permeability promote systemic inflammation, which, among other things, can increase blood-brain barrier permeability (3a). Intestinal inflammation, systemic inflammation, and synuclein pathology in the brain all promote neuroinflammation (4) which drives the neurodegeneration that characterizes PD (5).

1.2 Regulator of G-Protein Signaling 10

As peripheral and neuroinflammation are likely involved in the pathogenesis and progression of Parkinson's disease (PD), modulators of inflammation may play important regulatory roles that influence the risk for and dynamics of the development of parkinsonian pathology. One such factor is the Regulator of G-Protein Signaling 10 (RGS10).

1.2a RSG10 biochemistry

The family of proteins known as regulators of G protein signaling (RGS) includes numerous molecules that play vital roles in an array of biological processes. The unifying feature in this protein family is a RGS domain that has been conserved in eukaryotes ranging from single-celled yeast to humans (253). This domain exhibits guanosine-5'-triphosphatase (GTPase)-activating functionality which serves to terminate signaling downstream of activated G protein-coupled receptors (GPCRs). As such, RGS proteins are characterized as GTPase-activating proteins (GAPs). RGS10 is one of the smallest known RGS proteins at 21 kDa, and it has no recognized functional domain besides the RGS domain, but it has been reported to exert significant influence in a variety of systems and to impact inflammatory immune responses and parkinsonian pathology.

The *RGS10* gene is found on chromosome 10 in humans and chromosome 7 in mice (254). Three splice variants of the RGS10 protein exist in humans, and two are present in mice. The variants differ by only a few amino acids in the N-terminus, but they exhibit pronounced differences in localization and function, with the shortest forms reported to have limited GTPase-activating functionality and to localize to the nucleus and perinuclear space, suggesting that RGS10's GAP activity may not be its only function (103, 254). RGS10 function and localization

can also be regulated by post-translational modification. Palmitoylation in the RGS domain induces localization to the cell membrane and enhanced GAP activity in vesicle-based assays (255, 256), whereas phosphorylation by protein kinase A (PKA) at serine 168 (S168) induces nuclear localization (257). Based on sequence homology in the RGS domain, RGS10 has been assigned to the D/R12 subfamily of RGS proteins (27, 103). It has been reported to exert GAP activity on $G\alpha_{i1/3}$, $G\alpha_o$, $G\alpha_q$, and $G\alpha_z$ proteins in different systems, but not on $G\alpha_s$ type proteins ((253), reviewed by (103)). RGS10 is expressed to varying degrees throughout the mammalian body, and with the flexibility provided by splice variants, protein modifications, and multiple interacting partners which may be involved in diverse signaling pathways in different cell types, it is not surprising that RGS10 is being identified as a key player in numerous physiological processes.

1.2b RGS10 in osteoclasts

One of the earliest reports of RGS10 function in a specific system focused on osteoclasts – cells that degrade bone. In 2007, Yang *et al.* reported that RGS10 was highly expressed in human osteoclastoma. To probe this finding, they developed an RGS10 knockout (KO) mouse model in which they observed impaired osteoclast differentiation and severe osteopetrosis that was typically fatal within the first few weeks of life. They reported that, in this model, RGS10 associated competitively with calmodulin and phosphoinositol triphosphate (PIP_3), resulting in activation of phospholipase C gamma (PLCγ) and altered Ca^{2+} oscillations (258).

The association of these severe skeletal phenotypes with RGS10 has not been corroborated, and there is reason to speculate that the observed deficiencies in the knockout animals were not primarily caused by the loss of RGS10 expression, but rather by inadvertent

disruption during homologous recombination of genes involved in the fibroblast growth factor (FGF) and transforming growth factor (TGF) signaling pathways near the *Rgs10* locus. In mice, four *Fgf* genes are located on the same chromosome as *Rgs10*, and the *Fgfr2* gene is situated in close proximity on the same strand. Defects in FGF signaling have been reported to cause myriad skeletal abnormalities of the type reported by Yang *et al* ((259), reviewed by (260)). *Tgfb1* is also located on mouse chromosome 7, and *Tgfb1i1* which encodes TGFβ1 induced protein 1, a regulator of TGFβ activity, is closely juxtaposed with *Rgs10* exons. TGFβ signaling has been implicated in bone remodeling, and it has even been reported to inhibit osteoclasts, another function attributed to RGS10 in the 2007 study (261-263).

While any involvement of FGF or TGFβ deficiencies in the phenotypes of the RGS10 KO mice described by Yang *et al.* (258) is only speculative, it is reasonable to consider when such a profound phenotype is observed after disrupting the chromosomal region containing well-established mediators of skeletal formation. Lending support to this theory, RGS10-null mice generated using CRISPR/Cas (264) or exon trapping (265) technologies rather than conventional exon deletion exhibit no gross bone abnormalities or shortened lifespans. It may also be worth noting that the RGS10 KO mice referenced in the Yang *et al*. study have not been used in subsequent studies on RGS10 published by that research group.

In spite of the questions surrounding the initial study of RGS10's function in osteoclasts, RGS10 may still play a role in bone development and repair. Another study by Yang *et al*. seemed to corroborate some of their *in vivo* findings using RNA interference to silence expression of one splice variant of RGS10 (266). They reported that RGS10 expression was induced by receptor activator of nuclear factor kappa-B ligand (RANKL) in osteoclast precursors, and that reductions in RGS10 inhibited NFAT2 expression, intracellular Ca^{2+}

oscillations, and osteoclast differentiation from bone marrow cells and osteoclast precursor cell lines (266). Notably, osteoclasts differentiate from monocytes and macrophages, and there is abundant evidence that RGS10 significantly influences the function and responses of macrophages and related cells (265, 267-269), so it is possible that the observations concerning osteoclast development may be related to RGS10's impact on the inducibility of these osteoclast precursors.

1.2c RGS10 in cancer

More recent studies have begun to explore the role of RGS10 in cancerous cells. In ovarian cancer cell lines, RGS10 expression was found to be reduced in response to cytotoxic chemotherapy (270). The downregulation was mediated by epigenetic modifications, specifically higher expression of histone deacetylase 1 (HDAC1) and DNA methyltransferase 1 (DNMT1) that resulted in enhanced methylation of the *RGS10* promoter and reduced acetylation of histone H3 (271, 272). The reduction in RGS10 promoted survival of the cancer cells, reducing their sensitivity to chemotherapy (270). Similar suppression of RGS10 was observed in hepatocellular carcinoma cells (273). RGS10 has been associated with inhibition of Akt activation (270) and GAP activity limiting the capacity of the small GTPase Rheb to activate mTOR (274), and so suppression of RGS10 expression could promote cell survival. Furthermore, the production of prostaglandins stimulated by cyclooxygenase-2 (COX2) is known to mediate chemoresistance in ovarian cancer cells, and reductions in RGS10 have been shown to increase COX2 levels in these cells by a mechanism unrelated to GAP activity, providing another pathway by which loss of RGS10 could promote chemoresistance (275).

Another study in laryngeal cancer cell lines reported the opposite finding, however, with RGS10 expression increased in chemoresistant cells (276). *RGS10* was also found to be significantly up-regulated in pediatric acute myeloid leukemia, and its levels were associated with poor clinical prognosis (277). These dissimilarities may be attributed to differences in RGS10's function in different cell types or to differences in the response of cells to various cytotoxic agents, and further research will be required to define the nature and extent of RGS10's impact in cancer.

1.2d RSG10 in neurotransmission

Some of the earliest reports on RGS10 noted its prominent expression in the brain (254), and the role of this protein in regulating neurological activity is an active area of research. RGS10 has been reported to inhibit $G\alpha_i$- and $G\alpha_q$-linked signaling through the 5-HT1A serotonin receptor but not through the D2 dopamine receptor when the receptors were stably expressed on CHOK1 cells (278). Similarly, RGS10 was identified as an inhibitor of μ opioid receptor but not δ opioid receptor signaling when the receptors were expressed on HEK293 cells (279). It has also been reported that RGS10 inhibits $G\alpha_{i/o}$ signaling involved in the response to antiepileptogenic low-frequency stimulation of the perforant path (280). These inhibitory functions seem to result from RGS10's established GAP activity, but a potentially non-canonical role for RGS10 was also reported in the CHOK1 cells system; RGS10 inhibited AC activity induced by forskolin, which does not act through G-protein signaling cascades (278).

Beyond these basic mechanistic findings, RGS10's impact on neurological processes is more cryptic. This protein has been shown to be regulated by DNMTs during human neural progenitor differentiation, but its role in neuronal development is unclear (281). A polymorphism

in the RGS10 gene was identified in a study of schizophrenic patients (282), but efforts to substantiate these findings at the protein level have revealed no differences in expression between affected individuals and healthy controls (283). Acute monoamine depletion in response to reserpine, a drug occasionally prescribed for schizophrenic patients, increased RGS10 levels in the striatum, but levels decreased with extended treatment, and the consequences of these fluctuations in RGS10 levels are unknown (284). RGS10 has also been linked to drug abuse and addiction, as RGS10 levels decrease in the prefrontal cortex with short-term opiate abuse in humans (285) and in the ventral tegmental area with chronic self-administration of amphetamines in rats (286). The authors of the amphetamine study suggested that the regulation of RGS10 might be connected to the diminishing expression of dopamine D2/D3 receptors that accompanies escalating psychostimulant use (286). Details of this regulatory mechanism and the functional impact RGS10 may have on responses to psychoactive drugs remain to be elucidated.

1.2e RGS10 in neuroinflammation and Parkinson's Disease

There is also evidence that suggests that RGS10 may be relevant in Parkinson's disease and specifically that it may mitigate parkinsonian pathology in the central nervous system (CNS). It has been well established that dopaminergic neuron degeneration in PD occurs on a backdrop of chronic inflammation, with persistent microglial activation and elevated levels of proinflammatory cytokines such as tumor necrosis factor (TNF) in the CNS as well as the periphery. These inflammatory mediators may be responsible for some of the neuron loss observed in PD. When a dopaminergic cell line (MN9D) was exposed to TNF or lipopolysaccharide (LPS) in models of inflammation-induced neurodegeneration, RGS10 expression in these cells decreased significantly, and the reduction promoted the death of the

cells (287). Deliberate siRNA knockdown of RGS10 in MN9D cells increased their sensitivity to the cytotoxic effects of treatment with conditioned media from LPS-activated microglia (265), and overexpression of RGS10 protected MN9D cells from TNF- (287) and oxidative stress-mediated (103) cytoxicity. This occurred through modulation of cell survival signaling, as was reported in some cancer studies, including increased expression of the pro-survival molecule Bcl-2 which inhibits caspase-mediated apoptosis (287).

RGS10 is also expressed in microglia, resident immune cells of the central nervous system which closely resemble peripheral macrophages (265). Exposure to sphingosine 1-phosphate (S1P) enhances *Rgs10* expression in microglia, and exposure to lipopolysaccharide (LPS) suppresses it (265, 288). This is mediated by the same HDAC regulatory pathway described in ovarian cancer cells (288). RGS10 is a potent regulator of microglial inflammatory activity. Both primary microglia from RGS10-null mice and cells from the BV2 microglial line in which RGS10 expression had been reduced with siRNA exhibited dysregulated expression of numerous genes including many encoding cytokines, chemokines, and their regulators at baseline and in response to LPS. When deficient in RGS10, both cell types produced more TNF, Fas, IL-1β, and other cytokines (265, 268, 275) and also exhibited less phagocytic activity (268), suggesting that they adopted a more proinflammatory and cytotoxic phenotype. This was confirmed when RGS10-deficient BV2 cells and RGS10-null microglia stimulated with LPS proved more toxic to a dopaminergic cell line than WT microglia, an effect primarily mediated by their increased production of TNF (265, 268). RGS10 may also contribute to anti-inflammatory activities in astrocytes as well, as low doses of carbon monoxide which reduce inflammation and promote cell survival stimulate the expression of *Rgs10* in primary murine astrocytes (289).

In vivo, RGS10-null mice on a mixed 129/C57BL/6J background exhibited increased microglial density and activation in the brain compared to WT at baseline and after chronic dosing with LPS. Most intriguingly, when RGS10-null mice were repeatedly exposed to low dose intraperitoneal LPS, they developed a parkinsonian phenotype with a pronounced loss of dopaminergic neurons in the substantia nigra (SN) and greater atrophy and degeneration of the remaining neurons, while minimal pathology was observed in similarly-treated WT mice (265). When RGS10 was overexpressed in microglia in the ventral midbrain of rats treated with neurotoxic OHDA, it prevented microgliosis and reduced the loss of dopaminergic neurons (268).

Thus, studies have described two separate but related pathways by which RGS10 may protect dopaminergic neurons from PD-like degeneration – by limiting the cytotoxic proinflammatory activity of CNS immune cells and by decreasing the sensitivity of dopaminergic neurons to inflammation-induced cell death.

1.2f RGS10 in peripheral immune cells and inflammation

These findings regarding RGS10's anti-inflammatory potential in the CNS fit neatly into a growing body of literature demonstrating RGS10's potent immunomodulatory activity. Numerous reports have confirmed the expression of RGS10 in peripheral immune-related tissue, and more specifically in lymphocytes (254, 290, 291), dendritic cells (DCs) (292), and macrophages, where it is expressed more abundantly than any other RGS protein (267). RGS10 has been shown to limit proinflammatory activity in macrophages as it did in microglia. When stimulated with LPS, macrophages derived from RGS10-null mice secreted higher levels of TNF, IL-1, IL-6, IL-12p70, and IL-10 (267), as microglia did, indicating that they more readily

adopted a classic inflammatory phenotype. Consistent with this altered regulation, RGS10-deficient macrophages exhibited a blunted response to alternative activation via IL-4 priming and TLR-4 stimulation (267). As with microglia, conditioned media from LPS-treated RGS10-null macrophages induced more death of MN9D cells than media from stimulated WT macrophages (267).

When efforts were made to identify a mechanism by which RGS10 mediated its anti-inflammatory effects in macrophages, it was determined that RGS10-null macrophages stimulated with LPS exhibited increased expression and phosphorylation of the p65 subunit of the transcription factor NFκB (267). The inhibition of NFκB acitivity by RGS10 would explain the exaggerated proinflammatory responses observed in RGS10-null macrophages, as NFκB is a key mediator of immune activation and regulates the transcription of numerous cytokine genes and other inflammatory response elements. It is likely that this same mechanism of NFκB inhibition accounts for the observances in microglia as well, as the loss of RGS10 in microglia resulted in higher expression of NFκB p65 and p50 and enhanced NFκB activity in response to TNF and LPS stimulation (268). No direct interactions between RGS10 and NFκB have been demonstrated, suggesting that RGS10 either acts upstream of NFκB in a signaling pathway or interferes with NFκB's expression or transcription factor activity in some way. The latter may be the more likely as RGS10 has been shown to translocate to the nucleus following exposure to stimuli that activate NFκB (265), and a study reporting on increased production of TNF and COX2 – both transcriptionally activated by NFκB – in RGS10-null microglia found that this effect was independent of GAP activity (275).

Another mechanism by which RGS10 can alter immune activity is by regulating the chemotactic responses of T cells. T cells are recruited to enter a tissue by binding chemotactic

molecules. As chemokines signal through heterotrimeric G protein cascades, RGS proteins may be considered likely candidates for regulation of these pathways. Specifically, RGS10 has been reported to inhibit adhesion of T cell lines to endothelial cells in response to $G\alpha_i$-dependent signaling of the chemokines CXCL12 and CCL21 (291). RGS10 inhibited the Vav1-Rac1 pathway, limiting the strengthening of adhesive integrin-mediated interactions between T cells and the endothelium. Furthermore, it was demonstrated that RGS10 knockdown in T cells induced sustained activation of Cdc42, a small GTPase which regulates actin polymerization in chemotaxis (291). These findings suggest that RGS10 limits T cell chemotaxis, consistent with other findings characterizing RGS10 as an anti-inflammatory regulator.

Several recent studies also broadly corroborate the negative associations between RGS10 and inflammation. A single nucleotide polymorphism in the *RGS10* locus was linked to increased risk for depression (293); inflammation is considered a primary driver of depression symptoms in a subset of patients and a common occurrence in most (294). Platelets are actively involved in inflammatory responses, and RGS10 deficiency enhances the activation and thrombogenic activity of platelets, at least in part through its GAP functionality (295, 296). RGS10 levels are suppressed in human and murine failing and hypertrophic heart tissue, and cardiac pathology is more severe in RGS10-null and less severe in RGS10-overexpressing mice (264). This may be attributed to RGS10's inhibitory effects on NFκB (297) and mTOR (298) as well as abrogation of GPCR signaling (264). Finally, RGS10-null mice fed a high-fat diet developed a more severe metabolic syndrome characterized by increased inflammation, insulin resistance, and weight gain compared with WT high-fat diet-fed controls (299).

Seemingly contradictory findings in other studies suggest that RGS10's regulatory role in inflammation and chemotaxis may be more complex and not universally anti-inflammatory. In a

Porphyromonas gingivales infection model of periodontitis, it was reported that local knockdown of RGS10 expression by adeno-associated virus(AAV)-mediated RNAi one day after *P. gingivales* infection protected against bone loss, reduced the number of osteoclasts in the tooth root, limited inflammatory cytokines and inflammation-mediated tissue damage, and decreased the number of T cells and DCs in the periodontal ligament 55 days after infection (300). RSG10-null mice were also reported to experience less frequent and severe experimental autoimmune encephalomyelitis (EAE) (301). Peripheral immune cells from these $RGS10^{-/-}$ animals exhibited blunted recall responses to the immunogen myelin oligodendrocyte protein, and fewer T cells and CD11b+ myeloid cells were found in the CNS (301). Adoptive transfer experiments indicated that T helper type 1 (Th1) cells were the mediators of the milder EAE, not Th17s (301). While these findings do not readily align with reports of RGS10's anti-inflammatory actions, it may be that, as has been suggested by studies of RGS10's role in other physiological processes, the function of this protein varies in different cell and tissue types. The activity of RGS10 could also differ in settings of acute infection, immunization, or autoimmunity compared with the sterile inflammation and cytokine- or TLR ligand-induced stimulation employed in other studies. Further investigation will be required to establish the function of RGS10 in different inflammatory conditions in humans, animal models, and *in vitro* systems.

1.2g RSG10 in aging

As RGS10 has evident immunomodulatory activity, and as the immune system changes significantly with age, it is worthwhile to consider that RGS10 may have unique functionality in an aging system. For instance, the chromosomal region containing *RGS10* has been linked to age-related maculopathy (ARM) (302), but no differences in expression of *RGS10* have been

found in dermal fibroblasts from humans with the disease. It is possible that RGS10 levels may be modulated in ARM-affected tissue, or its function may be impacted regardless of its expression. A detailed analysis of RGS10 in the brain and immune tissues of young and aged C57Bl/6J mice indicated that RGS10 levels increased with age in peripheral blood B cells, monocytes, and granulocytes, did not change in the ventral midbrain and striatum, and decreased with age in microglia (269). This study also demonstrated that the absence of RGS10 impacted immune cell frequencies differently depending on age. Young RGS10-null mice had reduced frequencies of $CD8^+$ splenocytes compared to WT, while aged RGS10-null mice had reduced frequencies of $CD4^+$ T cells and monocytes in the spleen. The absence of RGS10 reduced the frequency of monocytes/microglia and increased the frequencies of granulocytes and $CD8^+$ T cells in the brains of young mice, but not aged. On the other hand, aged RGS10-null mice exhibited reduced frequencies of B cells and $CD4^+$ T cells in the peripheral blood, while young mice did not (269). These findings demonstrate that RGS10 impacts immune cell populations throughout the body and that its influence changes with age, providing further evidence of the complex functional spectrum occupied by this regulatory protein and supporting its potential involvement in age-related immunopathology.

Overall, there is general consensus in the published literature that RGS10 contributes to the regulation of immune function, cell survival, and neurological activity, all of which play fundamental roles in PD pathology, and this protein has been linked to parkinsonian conditions in animal models. While it remains to be established whether abnormalities or altered expression of RGS10 are present in PD in the brain or peripheral tissues of human patients and whether these might contribute to disease, the well-characterized immunomodulatory and neuroprotective activity of RGS10 in specific cell types suggests that RGS10-null animal models could serve as

valuable tools to examine the contributions of inflammation to parkinsonian pathology. Determining whether intestinal inflammation is a hallmark of PD and by what mechanisms and under what conditions it could induce PD-related neurological impairments are fundamental questions necessary to understand the involvement of the gut-brain-immune axis in this disorder and how the activity of molecular regulators of the gut-brain-immune axis could be targeted to treat and prevent the clinical manifestations of this disease.

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CHAPTER 2: STOOL IMMUNE PROFILES EVINCE GASTROINTESTINAL INFLAMMATION IN PARKINSON'S DISEASE

Notes: This chapter has been previously published (1). All figures and tables represent data based on samples processed by the dissertation author, collected by the Emory Multiplexed Immunoassay Core, and analyzed by the dissertation author in collaboration with biostatistician Vicki Hertzberg, Ph.D.

2.1 Introduction

Parkinson's disease (PD) is currently defined by motor impairments such as resting tremor, bradykinesia, rigidity, and gait disturbance, but non-motor features including cognitive impairment, hyposmia, anxiety, depression, sleep disturbances, and, prominently, gastrointestinal (GI) dysfunction have been gaining increasing attention and have a profound impact on quality of life. Constipation is reported by approximately 50% of PD patients (2) and can be detected by objective measures in nearly 80% (3). It frequently precedes the onset of motor symptoms by more than 15 years (4). Pathological abnormalities including the PD-related aggregation of alpha synuclein (αSYN) have been identified in intestinal biopsies from PD patients (5) and even in subjects in the pre-motor phase of disease (6, 7), although consensus on the best interpretation of these results has not been reached (reviewed in (8)). PD patients also exhibit significant differences in gut bacterial populations (9-21) and increased intestinal permeability compared to individuals without PD (22, 23). With accumulating evidence that GI symptoms are present from the earliest stages of PD, it has been proposed that PD pathology may originate in the gut and later spread to the central nervous system (CNS). Immune activity may advance this progression,

as intestinal inflammation can promote systemic and also CNS inflammation (24, 25) which contributes to PD-related neurodegeneration (26).

Intestinal symptoms observed in PD are consistent with conditions of inflammation. Constipation in PD patients is attributed to slow intestinal transit time (27, 28), a symptom which is also frequently observed in other conditions involving chronic GI inflammation such as inflammatory bowel disease (IBD) (29) and obesity (30). Mechanistically, this condition has been linked to intestinal immune activation (31) and to intestinal dysbiosis, which is another hallmark of disorders involving gut inflammation (29, 32). Immune activation promotes increased αSYN expression and aggregation (22, 33, 34), and αSYN in turn stimulates proinflammatory immune responses (33, 35). In keeping with the well-known connections between inflammatory stimuli and impairment of gut barrier function (22, 36), intestinal permeability in PD correlates with levels of αSYN as well as indicators of oxidative stress (23). Additionally, recent studies have reported significant coincidence of IBD or irritable bowel syndrome and PD (37-39). One study reported the most direct confirmation of intestinal inflammation in PD, finding increases in mRNA transcripts encoding four proinflammatory cytokines (tumor necrosis factor, interferon gamma, interleukin-6, and interleukin-1β) as well as three glial markers in colonic biopsies of PD patients compared to age-matched healthy controls (40).

Given the accumulation of evidence suggesting that pronounced GI dysfunction and dysbiosis occurs in PD, we conducted an extensive analysis of immune and angiogenesis factors in stool to assess the GI inflammatory state in PD patients, their healthy spouses, and unrelated healthy control subjects. We also evaluated the prevalence of certain PD-associated non-motor

symptoms in our subject cohort and assessed how these and other physiological and lifestyle factors influenced levels of stool analytes.

2.2 Methods

2.2a Subjects

A subset of the NeuroGenetics Research Consortium subjects from Atlanta, GA; Albany, NY; and Seattle, WA were invited to participate (re-contacted based on prior Institutional Review Board-approved consents). These included most of the subjects evaluated for stool microbiota composition described by Hill-Burns *et al* (17), excluding those for whom stool samples were of insufficient quantity for this analysis. Of 266 subjects, 156 had been diagnosed with PD by a movement disorder specialist (PD patients), and 110 reported no diagnosis of PD (controls). Within the control group, 49 subjects were spouses of PD patients (household controls), 39 of whom had a spouse participating in this study, and 61 were not known to live with anyone diagnosed with PD (non-household controls). We evaluated whether any significant differences existed between household and non-household control groups in levels of stool analytes (**Table 1**) or in various relevant metadata factors (**Table 2**), and, finding none, we combined these groups for all analyses except a paired comparison of PD patients and their respective spouses. Over 99% of subjects described their race as "white." Ages of participants ranged from 36 to 94 years; mean age of control subjects was 70.8 years (standard deviation 8.8 years), and mean age of PD subjects was 68.3 years (standard deviation 8.8 years). A strong male sex bias was observed in PD subjects, with 112 males and 44 females. Accordingly, the opposite sex bias was observed in household controls, with 36 females and 13 males. Non-household controls consisted of 35 males and 26 females. In addition to providing a stool

sample, subjects completed a questionnaire on demographics, health problems, medications, and dietary practices.

2.2b Processing of stool

Stool samples were collected on BBL CultureSwabs (Becton, Dickson and Company; Sparks, MD) by participants at home, shipped immediately via the United States Postal Service at ambient temperature, and then stored at -20°C until processing. Swab tips were placed in tubes with 5-mm stainless steel beads (QIAGEN, Valencia, CA) and 550µL homogenization buffer (125mM Tris, 15mM MgCl2, 2.5mM EDTA pH 7.2, 1% Triton X-100, 1 tablet protease inhibitors [1697498, Roche, Indianapolis, IN] per 10mL buffer). Samples were agitated (20Hz) in chilled racks in TissueLyser II (QIAGEN) for three cycles of two minutes. Debris were pelleted by centrifugation, and supernatants were collected.

2.2c Multiplexed immunoassays

Levels of immune factors were measured in 40µL undiluted stool homogenates using the V-PLEX Neuroinflammation Panel 1 (human) Kit (Meso Scale Discovery, Rockville, MD) according to the manufacturer's protocol. This kit is divided into 5 panels of analytes grouped into the physiological categories "Angiogenesis," "Chemokine," "Cytokine," "Proinflammatory," and "Vascular Injury." Analytes measured were: vascular endothelial growth factor receptor 1 (Flt1), placental growth factor (PIGF), tyrosine kinase 2 (Tie-2), vascular endothelial growth factor A (VEGF), vascular endothelial growth factor D (VEGF-D), basic fibroblast growth factor (bFGF), eotaxin (CCL11), eotaxin-3 (CCL26), interferon gamma-induced protein 10 (IP-10, CXCL10), monocyte chemoattractant protein 1 (MCP-1, CCL2),

monocyte chemoattractant protein 4 (MCP-4, CCL13), macrophage-derived chemokine (MDC, CCL22), macrophage inflammatory protein 1 alpha (MIP-1α, CCL3), macrophage inflammatory protein 1 beta (MIP-1β, CCL4), thymus and activation-regulated chemokine (TARC, CCL17), interleukin 12/interleukin 23 p40 (IL-12/IL-23 p40), interleukin 15 (IL-15), interleukin 16 (IL-16), interleukin 17 A (IL-17A), interleukin 1 alpha (IL-1α), interleukin 5 (IL-5), interleukin 7 (IL-7), lymphotoxin alpha (LTA), vascular endothelial growth factor C (VEGF-C), interferon gamma (IFNγ), interleukin-10 (IL-10), interleukin-13 (IL-13), interleukin-1 beta (IL-1β), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-8 (IL-8, CXCL8), tumor necrosis factor (TNF), C-reactive protein (CRP), serum amyloid A (SAA), soluble intercellular adhesion molecule 1 (sICAM-1), soluble vascular cell adhesion molecule 1 (sVCAM-1).

Analyte levels were measured on the Meso Scale Discovery (MSD) QuickPlex instrument and evaluated on the MSD software platform. Values were normalized to total protein measured in each sample by Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) according to manufacturer's protocol.

2.2d Statistical analysis

Chi square tests (two-tailed) were utilized to assess relationships between disease status (PD patients/controls) and discrete-valued (e.g., Yes/No) responses to various health-related questions. T-tests were used to compare levels of stool analytes between PD patients and controls, and Wilcoxon rank sum test was used to compare levels of stool analytes between males and females (t-test deemed inappropriate due to data skew). General linear models (GLM) were used to investigate relationships between disease status and metadata factors. Multivariate

analysis was performed using multiple regression GLM with multiple imputation (5) with the following potential confounders or effect modifiers: age, sex, geographic location, antibiotic use in the three months prior to stool collection, use of anti-inflammatory drugs which in these subjects included non-steroidal anti-inflammatory drugs or prednisone at least thrice a week (included automatically); body mass index (BMI), smoked at least 100 cigarettes in lifetime, current probiotic use including probiotic supplements or yogurt with live culture (included due to strong effects on stool analyte levels); birth by Caesarean section, digestive problems on the day of stool collection, diarrhea in the three months prior to stool collection, constipation in the three months prior to stool collection, coffee consumption, and alcohol consumption (included because incidence differed significantly between PD patients and controls). Unless otherwise specified, analyses were conducted using PROC GLM and PROC MIXED in SAS/STAT® software v9.3 for PC (SAS Institute Inc. 2011, SAS/STAT® 9.3 User's Guide. Cary, NC: SAS Institute Inc).

2.3 Results

2.3a Increased incidence of psychological and gastrointestinal symptoms in PD patients and decreased coffee and alcohol consumption

As expected, PD patients in this study reported increased incidence of anxiety (11 Controls-10.0%, 41 PD-26.1%, X^2=11.52, p=0.0007), depression (24 Controls-21.8%, 52 PD-33.1%, X^2=5.147, p=0.0233), and sleep problems (14 Controls-12.7%, 61 PD-38.9%, X^2=22.61, p=0.0001) (**Table 3**). Intestinal disease, which included inflammatory bowel disease, irritable bowel syndrome, Crohn's disease, and colitis, was also more common among PD patients (9 Controls-8.2%, 26 PD-16.6%, X^2=4.390, p=0.0361) (**Table 3**). There was no difference in subjects' reports of ulcers (**Table 4**). Over 60% of PD patients who responded to the question

reported experiencing digestive problems in the three months prior to stool collection, significantly more than controls (40 Controls-36.4%, 101 PD-64.3%, X^2=22.69, p<0.0001) (**Table 3**). This difference was driven by increased incidence of constipation in the past three months (4 Controls-3.6%, 65 PD-41.4%) as well as increased incidence of bloating (2 Controls-1.8%, 15 PD-9.6%) and excessive gas (2 Controls-1.8%, 21 PD-13.4%) on the day of stool collection, and it persisted despite decreased incidence of diarrhea over three months (27 Controls-24.5%, 21 PD-13.4%). PD patients were also more likely than controls to report taking medication for digestive problems (16 Controls-14.5%, 47 PD-29.9%, X^2=8.086, p=0.0045) (**Table 3**).

Among our subjects, there was a significant association between reported coffee consumption and disease status (X^2=11.61, p=0.0205), with controls reporting greater coffee intake (median 1-2 cups a day) than PD patients (median 2-6 cups a week) (**Table 3**). The same pattern was observed for alcohol consumption (X^2=19.38, p=0.0016) (**Table 3**). No differences were found between PD patients and controls with regard to smoking history, use of anti-inflammatory drugs, antibiotics, or probiotics. There were also no significant dietary differences, although trends for reduced consumption of vegetables/fruits (X^2=5.365, p=0.0684) and nuts (X^2=7.353, p=0.0614) among PD patients were present (**Table 4**).

2.3b Significantly higher levels of select stool analytes in PD patients

We first performed a direct comparison of immune- and angiogenesis-related analyte levels in the stool samples from PD patients and controls. Mean levels of 34 of 37 factors were higher in patients than controls, but three, Flt1, IL-1α, and CXCL8, met the threshold for significance (p<0.05) (**Table 5**).

To better account for the effects of subjects' environments on stool analyte levels, we performed a paired comparison of PD patients and their respective non-PD spouses. When all PD patients were compared with all household controls by paired t-test, there were no significant differences in levels of immune or angiogenesis factors. When patient-spouse pairs were separated by patient sex, however, a different pattern emerged. Male PD patients still exhibited no significant differences compared to their female spouses, but female PD patients had higher levels of 11 cytokines, chemokines, and angiogenesis factors compared to male spouses (**Table 6**).

2.3c Sex significantly impacts stool analyte levels

The household control comparison suggested that subject sex greatly impacted levels of immune and angiogenesis factors in stool. Indeed, when analyte concentrations between female and male subjects were compared, females exhibited significantly higher levels of 21 of 37 factors (**Table 7**). The relationships between sex and disease status for each analyte were largely consistent, but three factors, PIGF, CXCL10 (IP-10), and CCL2 (MCP-1), were found at higher concentrations in female PD patients compared to female controls while no differences were found between male patients and controls (**Fig 2**).

2.3d BMI, smoking history, and probiotic usage strongly influence stool analyte levels

Other metadata variables also influenced stool analyte levels. Nine chemokines and angiogenesis factors decreased significantly with increasing subject body mass index (BMI) (**Fig 3**). Subjects who reported smoking at least 100 cigarettes in their lives (~5 packs) had significantly lower levels of eight different cytokines and vascular factors in stool (**Fig 4**),

regardless of disease status. One classically proinflammatory cytokine, IL-6, was significantly reduced with increasing coffee consumption (**Fig 5**). Consumption of probiotics or probiotic yogurt was associated with significantly higher levels of three analytes, TARC (CCL17), IL-7, and MIP-1β, regardless of disease status (**Fig 6a**), but an additional eight factors were significantly elevated only in PD patients taking probiotics (**Fig 6b**). Most of the analytes affected by probiotic use were chemokines.

2.3e PD-associated stool inflammatory profile emerges when other factors are accounted for

Given the profound impact of numerous metadata factors on levels of stool analytes, we decided to more specifically evaluate PD-associated inflammatory signatures using a multivariate GLM approach adjusting for 13 metadata parameters. In this analysis, PD disease status was significantly ($p<0.05$) associated with elevated levels of IL-1α, IL-1β, CXCL8, and CRP in stool. Elevations in Flt1 and PIGF were also trending ($p=0.0541$). (**Table 8**).

2.3f PD-associated GI inflammation does not emerge only in advanced disease

Alterations in immune activity occur with aging (41), and age is the primary risk factor for development of PD. Additionally, whether intestinal inflammation and dysfunction represent an independent facet of PD pathology or simply a response to increasing neurodegeneration remains unclear. To explore these potential relationships, we evaluated the effects of subject age and PD duration on the levels of immune and angiogenesis factors. A GLM regression of PD duration (age at time of stool collection minus age of PD onset) versus stool analyte levels revealed no significant association for any analyte (**Table 9**). Mean levels of immune and

angiogenesis factors remained consistent from recently diagnosed subjects to individuals who had lived with PD for more than 30 years. A GLM analysis evaluating the effects of subject age and disease status on stool analyte levels identified significant decreases in VEGF-C and IL-6 with increasing age regardless of disease status (**Fig 7**). Significant differences in the relationships between age and analyte levels for PD patients and controls were identified for MCP-1, TNF, IL-10, and IL-4. Levels of these factors trended toward a decrease with age in PD patients but remained relatively constant with age in controls (**Fig 7**). While not significant, this same pattern was observed for the majority of analytes measured.

2.4 Discussion

PD patients in this study reported the increased incidence of psychiatric and GI symptoms that is typical of PD. Measurements of stool immune factors indicated that gastrointestinal inflammation is present in these PD patients. This supports and expands upon previous reports of PD-associated dysbiosis, GI dysfunction, and colonic inflammation.

When levels of stool analytes were directly compared in PD patients and controls, Flt1, IL-1α, and CXCL8 were found to be significantly elevated. PD-associated increases in CXCL8 and IL-1α persisted after adjusting for 13 potential confounders or effect modifiers. CXCL8 is a highly proinflammatory molecule with neutrophil chemoattractant as well as angiogenic properties (42). IL-1α is a potent initiator of GI inflammation which triggers recruitment of myeloid cells and their production of additional proinflammatory mediators such as IL-1β (43). Production of IL-1α and IL-1β are induced in inflammation in part through activity of the transcription factor NF-κB, and, in an amplification loop, they also stimulate NF-κB activity (43). In fact, all the inflammatory factors identified in the multivariate analysis as elevated in PD

patients – CXCL8, IL-1α, IL-1β, and CRP – are targets for NF-κB transcriptional activity (44-46), and, like IL-1 cytokines, CRP also promotes NF-κB activity (47). Our results suggest that this immune signaling pathway may be dysregulated in PD, leading to excessive inflammation.

In the multivariate analysis, elevations in angiogenesis-promoting Flt1 (VEGF receptor 1) as well as PIGF in stool from PD patients were trending ($p=0.0541$) but not significant. Inflammation and angiogenesis are closely linked (48) and frequently co-regulated, for instance by NFκB (49). Increased pro-angiogenic factors including VEGF and PIGF have been reported in cerebrospinal fluid from PD patients, and it has been suggested that this promotes the blood-brain barrier dysfunction that has been documented in PD (50, 51). Further investigation will be needed to determine whether consistent increases in angiogenic factors are present in the intestine in PD patients and how these might contribute to GI pathology.

The fact that disease-associated patterns in levels of immune factors did not change with PD duration suggests that intestinal inflammation is not exclusively present in advanced disease. PD-associated increases in stool analytes were also not explained by advanced subject age, as, if anything, levels of most analytes trended toward a decrease with age in PD patients. These findings support the hypothesis that intestinal inflammation is an early manifestation of PD that could contribute to the development of neuropathology rather than an effect arising in response to extensive gastrointestinal neurodegeneration.

Direct comparison of levels of stool immune and angiogenesis mediators in PD patients and controls was complicated by other variables. For instance, females were found to have significantly higher levels of most analytes measured compared to males. The minimal number of significant sex-disease status interactions, however, suggests that there is little sex difference in the nature of the impact that PD has on the gut immune environment. Higher levels of immune

factors in stool from female subjects, though, could possibly contribute to differences in the severity of symptoms in PD (52, 53).

Subject sex also influenced the paired comparison of PD patients with their non-PD spouses, with no differences found between male patients and their female spouses but significantly elevated levels of 11 cytokines, chemokines, and angiogenesis factors in female patients compared to their male spouses. If there were no differences in levels of stool immune and angiogenesis factors between patients and controls, then females should have exhibited significantly higher levels of many analytes regardless of disease status. Instead, only female PD patients exhibited significant elevations compared to their partners, while the levels of stool cytokines, chemokines, and angiogenesis factors in males with PD resembled their female spouses. This supports a disease-associated increase in numerous immune and angiogenesis mediators that persists in a shared environment.

We also observed several interesting patterns in lifestyle factors that can alter stool immune and angiogenesis factor levels. While there were no significant differences reported in diet between PD patients and controls, the trending reductions in fruit and vegetable and nut consumption in PD patients may warrant further investigation, particularly as the immunomodulatory and neuroprotective potential of these foods in the context of neurodegenerative disease is beginning to emerge (54, 55). The inverse correlations between BMI and certain stool analyte levels identified in this study may also merit further inquiry. While increased levels of immune and angiogenesis factors have been reported in serum and adipose tissue of overweight and obese subjects (56, 57), reductions in serum bFGF and Flt-1 with increasing BMI, such as we observed in stool, have been found (58, 59). Furthermore, while increased fecal calprotectin in obese subjects suggests intestinal inflammation (60, 61), the

relationship between BMI and levels of other immune and angiogenesis mediators in stool has not been explored, and our results suggest that it is complex.

Coffee and alcohol consumption and smoking are all reportedly reduced in PD patients (62-66), but whether these findings indicate protective effects of these practices, disease-associated suppression of psychological reward mechanisms (64), or simply reduced fluid intake due to dysphagia in advanced disease (67) remains undetermined. We observed reduced current coffee intake in our cohort of PD patients as well as reduced current alcohol consumption, in agreement with previous reports. While coffee and caffeine are known to have potent neuroprotective and anti-inflammatory properties (62), this study found only a minimal effect of alcohol and coffee consumption on levels of stool immune mediators, with coffee intake inversely associated with IL-6 levels. We found no differences between PD patients and controls in smoking history, but we did observe that having smoked at least 100 cigarettes reduced levels of multiple immune factors in stool. While cigarette smoke may have numerous deleterious effects systemically and in the gut (68-70), nicotine is known to have anti-inflammatory effects mediated by nicotinic acetylcholine receptor signaling and resulting in, among other effects, inhibition of NFκB (71). Our results indicate that this immunomodulatory activity is prominent in the human gut. Interestingly, the smoking-associated differences we observed persisted even though over 92% of respondents in this study who reported having smoked were not current smokers and had been non-smokers for an average of 36.6 years. This suggests that the modulation of intestinal immune function mediated by smoking may be at least semi-permanent, perhaps aided by lasting alterations in the microbiome (64) and by epigenetic modification (72).

The higher levels of chemokines and other inflammatory factors associated with probiotic use, especially in PD patients, could have several explanations. Probiotics are thought to

stimulate mucosal immune activity, and they may increase the production of chemokines that recruit primarily tolerogenic cell types. We also noted, however, that nearly 60% of controls and 80% of PD patients who reported using probiotics also reported experiencing digestive problems, so it is possible that the elevated levels of immune factors could result primarily from chronic GI problems rather than probiotics taken for their alleviation. A few studies have reported that the use of probiotics can ameliorate constipation in PD patients (73, 74); trials measuring biochemical as well as clinical responses to probiotics may clarify whether these are truly a beneficial treatment for individuals with PD. Furthermore, the reciprocal interactions between specific intestinal bacteria, whether transient probiotics or resident commensals, and intestinal immune responses in PD remain to be elucidated and will likely provide greater insight into mechanisms of disease pathology than studies of either factor in isolation.

Our study also corroborates the few reports (37-39) of associations between PD and intestinal disease, with significantly more PD patients than controls in our cohort reporting a history of inflammatory bowel disease, irritable bowel syndrome, Crohn's disease, and/or colitis. The mechanisms responsible for this epidemiological overlap have not been determined. There may be shared genetic predisposition for PD and intestinal disease; variations in the LRRK2 and NOD2 genes are associated with both PD (75, 76) and Crohn's disease (77-79). Another possibility is that the chronic inflammatory responses involved in GI diseases promote neuroinflammation and PD-associated neurodegeneration, a concept that is beginning to be tested in animal models (8, 24). Enteric inflammation and other changes in the GI environment in PD could also contribute to disruption of intestinal immune tolerance and trigger clinical intestinal disease.

This study provides evidence that classic inflammatory processes are overly active in the intestine in PD patients and do not arise only in advanced disease. These could promote systemic and neuroinflammation and, ultimately, parkinsonian neurodegeneration. Because the immune mediators found to be elevated in PD patients would be produced in response to diverse insults, their specificity as biomarkers for PD is limited. Prospective studies would also be needed to determine when these indicators of GI inflammation appear in relation to motor symptoms. However, in combination with key pieces of patient information, it is possible that levels of select immune factors in stool could enable identification of individuals at risk for development of PD. Understanding the connections between intestinal inflammation and systemic and neuroinflammation may yield new insight into the mechanisms of PD pathogenesis and guide future investigations into immunomodulatory therapy that could potentially slow progression of the disease.

Table 1. No differences in levels of immune or angiogenesis factors in stool from non-household and household controls

		NON-HOUSEHOLD CONTROLS			HOUSEHOLD CONTROLS			
Panel	**Factor**	**N**	**Mean (pg/mg)**	**Std Dev**	**N**	**Mean (pg/mg)**	**Std Dev**	**p-value**
Angiogenesis	**Flt1**	61	3.817	3.295	49	4.175	2.776	0.5462
	PlGF	61	1.376	0.648	49	1.325	0.7061	0.6950
	Tie2	61	178.9	99.7	49	186.3	109.5	0.7119
	VEGF-C	61	102.6	65.1	49	134.9	171.9	0.1784
	VEGF-D	61	18.73	9.82	49	20.37	13.06	0.4543
	bFGF	61	0.5911	0.4353	49	0.6550	0.4320	0.4442
Chemokine	**Eotaxin**	61	7.658	3.768	49	8.736	6.441	0.2759
	Eotaxin-3	61	21.02	21.16	49	24.34	20.42	0.4069
	IP-10	61	0.3841	0.2772	49	0.4166	0.2901	0.5506
	MCP-1	61	0.2614	0.3456	49	0.2529	0.1784	0.8765
	MCP-4	61	3.989	2.097	49	4.530	2.946	0.2638
	MDC	61	11.46	7.072	49	11.58	6.804	0.9282
	MIP-1α	61	4.186	3.336	49	4.640	3.440	0.4853
	MIP-1β	61	2.020	1.369	49	2.526	2.276	0.1514
	TARC	61	0.7553	0.5279	49	0.8125	0.6064	0.5977
Cytokine	**IL-12/23 p40**	61	2.519	1.698	49	2.449	1.300	0.8122
	IL-15	61	0.7449	0.6139	49	0.7696	0.6084	0.8342
	IL-16	61	15.62	16.23	49	15.03	11.36	0.8318
	IL-17A	61	2.032	1.486	49	2.213	1.425	0.5182
	IL-1α	61	43.33	38.4	49	39.10	43.24	0.5888
	IL-5	61	0.7127	0.6882	49	0.7054	0.6573	0.9551
	IL-7	61	0.6699	0.4438	49	0.7191	0.4079	0.5505
	LTA	61	0.4128	0.3202	49	0.4283	0.4357	0.8300
	VEGF	61	18.18	70.22	49	9.98	11.94	0.4216
Proinflammatory	**IFNγ**	61	10.05	15.67	49	9.39	13.13	0.8141
	IL-10	61	1.747	3.112	49	1.775	2.667	0.9602
	IL-13	61	3.262	1.742	49	3.275	2.065	0.9721
	IL-1β	61	1.826	2.54	49	1.540	2.457	0.5516
	IL-2	61	2.696	1.785	49	3.001	2.853	0.4945
	IL-4	61	0.5568	0.7229	49	0.6402	0.8082	0.5697
	IL-6	61	2.291	3.680	49	3.170	5.976	0.3452
	IL-8	61	17.5	69.7	49	10.1	21.2	0.4763
	TNF	61	0.7604	1.008	49	0.8114	1.0021	0.7920
Vascular Injury	**CRP**	61	560	1790	49	540.9	1376	0.9502
	SAA	61	74.7	58.8	49	68.76	45.93	0.5648
	sICAM-1	61	16.13	55.34	49	9.81	12.47	0.4353
	sVCAM-1	61	23.15	21.58	49	22.23	21.74	0.8265

Mean protein levels (pg/mg total protein) with standard deviations (Std Dev) in stool homogenates
N = number of measurements for each subject group
Gray shading indicates significant ($p<0.05$) difference between subject groups by t-test

Table 2. No differences between non-household and household controls in numerous demographic and health factors and practices

Question	Response	Subjects		X^2	p
		Non-household	*Household*		
Diagnosed or suspected anxiety	Yes	4	7	1.73	0.189
		6.6%	14.3%		
	No	56	42		
		91.8%	85.7%		
Diagnosed or suspected depression	Yes	15	9	0.603	0.438
		24.6%	18.4%		
	No	45	39		
		73.8%	79.6%		
Diagnosed or suspected sleep problems, insomnia	Yes	10	4	2.02	0.155
		16.4%	8.2%		
	No	47	45		
		77.0%	91.8%		
Experienced digestive problems in the past 3 months	Yes	21	19	0.0681	0.794
		34.4%	38.8%		
	No	32	26		
		52.5%	53.1%		
Currently on medication for digestive problems	Yes	6	10	1.68	0.196
		9.8%	20.4%		
	No	49	40		
		80.3%	81.6%		
Diagnosed or suspected IBD, IBS, Crohn's, or colitis	Yes	3	6	1.78	0.182
		4.9%	12.2%		
	No	57	44		
		93.4%	89.8%		
How much caffeinated coffee do you drink	None	9	17	8.66	0.0701
		14.8%	34.7%		
	<2 cups a week	8	2		
		13.1%	4.1%		
	2-6 cups a week	9	4		
		14.8%	8.2%		
	1-2 cups a day	18	17		
		29.5%	34.7%		
	3+ cups a day	15	9		
		24.6%	18.4%		
How much alcohol do you drink	None	14	17	6.72	0.243
		23.0%	34.7%		
	< 2 drinks a week	27	20		
		44.3%	40.8%		
	2-6 drinks a week	5	3		
		8.2%	6.1%		
	1 drink a day	7	2		
		11.5%	4.1%		
	2 drinks a day	5	7		
		8.2%	14.3%		
	3+ drinks a day	3	0		
		4.9%	0.0%		

Table 2 continued

Question	Response	Subjects		x^2	p
		Non-household	***Household***		
Smoked at least 100 cigarettes in your lifetime	Yes	22	15	0.0380	0.845
		36.1%	30.6%		
	No	31	23		
		50.8%	46.9%		
Completed a course of antibiotics in the past 3 months	Yes	10	7	0.179	0.672
		16.4%	14.3%		
	No	49	43		
		80.3%	87.8%		
Taking anti-inflammatory drugs at least 3 times a week	Yes	25	20	0.0267	0.870
		41.0%	40.8%		
	No	34	29		
		55.7%	59.2%		
Currently taking probiotic supplements or probiotic yogurt	Yes	14	13	0.112	0.738
		23.0%	26.5%		
	No	45	36		
		73.8%	73.5%		
Digestive problems on day of stool collection	Yes	6	6	0.285	0.594
		9.8%	12.2%		
	No	50	36		
		82.0%	73.5%		
Experienced constipation in the past 3 months	Yes	3	1	0.729	0.393
		4.9%	2.0%		
	No	56	49		
		91.8%	100.0%		
Experienced diarrhea in the past 3 months	Yes	14	13	0.0806	0.777
		23.0%	26.5%		
	No	44	36		
		72.1%	73.5%		
Subject location	Albany	21	27	4.73	0.0941
		34.4%	55.1%		
	Atlanta	7	4		
		11.5%	8.2%		
	Seattle	33	18		
		54.1%	36.7%		
		Mean	***Mean***	***t***	***p***
		Non-household	***Household***		
Subject Age		71.98	69.63	1.41	0.161
Subject Body Mass Index		27.97	28.7	0.717	0.475

Table 1. Increased incidence of psychological and gastrointestinal symptoms in PD patients and decreased coffee and alcohol consumption

Question	Response	Subjects		X^2	p
		Controls	*PD Patients*		
Diagnosed or suspected anxiety	Yes	11	41	11.52	0.0007
		10.0%	26.1%		
	No	98	110		
		89.1%	70.1%		
Diagnosed or suspected depression	Yes	24	52	5.147	0.0233
		21.8%	33.1%		
	No	84	95		
		76.4%	60.5%		
Diagnosed or suspected sleep problems, insomnia	Yes	14	61	22.61	0.0001
		12.7%	38.9%		
	No	92	89		
		83.6%	56.7%		
Experienced digestive problems in the past 3 months	Yes	40	101	22.69	<0.0001
		36.4%	64.3%		
	No	58	40		
		52.7%	25.5%		
Currently on medication for digestive problems	Yes	16	47	8.086	0.0045
		14.5%	29.9%		
	No	89	106		
		80.9%	67.5%		
Diagnosed or suspected IBD, IBS, Crohn's, or colitis	Yes	9	26	4.390	0.0361
		8.2%	16.6%		
	No	101	126		
		91.8%	80.3%		
How much caffeinated coffee do you drink	None	26	40	11.61	0.0205
		23.6%	25.5%		
	<2 cups a week	10	27		
		9.1%	17.2%		
	2-6 cups a week	13	16		
		11.8%	10.2%		
	1-2 cups a day	36	55		
		32.7%	35.0%		
	3+ cups a day	25	14		
		22.7%	8.9%		
How much alcohol do you drink	None	31	59	19.38	0.0016
		28.2%	37.6%		
	< 2 drinks a week	47	48		
		42.7%	30.6%		
	2-6 drinks a week	8	31		
		7.3%	19.7%		
	1 drink a day	9	7		
		8.2%	4.5%		
	2 drinks a day	12	5		
		10.9%	3.2%		
	3+ drinks a day	3	2		
		2.7%	1.3%		

Table 4. No differences between PD patients and controls in numerous health factors and practices

Question	Response	Subjects		X^2	p
		Controls	*PD Patients*		
Smoked at least 100 cigarettes in your lifetime	Yes	37	66	1.613	0.2041
		33.6%	42.0%		
	No	54	68		
		49.1%	43.3%		
Diagnosed or suspected ulcers	Yes	7	15	1.051	0.3053
		6.4%	9.6%		
	No	103	136		
		93.6%	86.6%		
Completed a course of antibiotics in the past 3 months	Yes	17	17	0.9645	0.3261
		15.5%	10.8%		
	No	92	132		
		83.6%	84.1%		
Taking anti-inflammatory drugs at least 3 times a week	Yes	45	61	0.02594	0.8720
		40.9%	38.9%		
	No	63	89		
		57.3%	56.7%		
Currently taking probiotic supplements or probiotic yogurt	Yes	27	34	0.06489	0.7989
		24.5%	21.7%		
	No	81	110		
		73.6%	70.1%		
How often do you eat grains	Few times a month or less	7	10	0.1035	0.9496
		6.4%	6.4%		
	Few times a week	26	39		
		23.6%	24.8%		
	At least once a day	74	101		
		67.3%	64.3%		
How often do you eat meat	Few times a month or less	3	9	1.5170	0.4683
		2.7%	5.7%		
	Few times a week	39	52		
		35.5%	33.1%		
	At least once a day	68	90		
		61.8%	57.3%		
How often do you eat fruit or vegetables	Few times a month or less	2	5	5.3650	0.0684
		1.8%	3.2%		
	Few times a week	11	30		
		10.0%	19.1%		
	At least once a day	97	117		
		88.2%	74.5%		
How often do you eat nuts	Once a month or less	7	21	7.3530	0.0614
		6.4%	13.4%		
	Few times a month	21	41		
		19.1%	26.1%		
	Few times a week	47	56		
		42.7%	35.7%		
	At least once a day	34	34		
		30.9%	21.7%		

Table 5. Comparison of levels of immune and angiogenesis factors in stool from PD patients and controls

		PATIENTS			CONTROLS			
Panel	**Factor**	**N**	**Mean (pg/mg)**	**Std Dev**	**N**	**Mean (pg/mg)**	**Std Dev**	**p-value**
Angiogenesis	**Flt1**	156	5.020	4.272	110	3.957	3.059	0.0184
	PlGF	156	1.439	1.132	110	1.352	0.6687	0.4328
	Tie2	156	194.2	152.1	110	181.3	103.7	0.4112
	VEGF-C	156	117.3	108.9	110	116.7	124.4	0.9662
	VEGF-D	156	19.49	14.50	110	19.39	11.32	0.9489
	bFGF	156	0.6937	0.5598	110	0.6165	0.4322	0.2040
Chemokine	**Eotaxin**	156	8.878	6.160	110	8.113	5.119	0.2697
	Eotaxin-3	156	24.05	19.06	110	22.35	20.77	0.4877
	IP-10	156	0.4657	0.4559	110	0.3964	0.2818	0.1265
	MCP-1	156	0.3225	0.5859	110	0.2562	0.2816	0.2195
	MCP-4	156	4.759	3.468	110	4.214	2.508	0.1366
	MDC	156	11.56	8.549	110	11.48	6.903	0.9306
	MIP-1α	156	4.879	4.204	110	4.363	3.370	0.2673
	MIP-1β	156	2.459	2.063	110	2.236	1.832	0.3616
	TARC	156	0.7362	0.6579	110	0.7790	0.5600	0.5781
Cytokine	**IL-12/23 p40**	156	2.634	1.779	110	2.481	1.522	0.4610
	IL-15	156	0.8354	0.6445	110	0.7519	0.6075	0.2857
	IL-16	156	16.34	17.50	110	15.28	14.17	0.5848
	IL-17A	156	2.955	8.555	110	2.104	1.452	0.2231
	IL-1α	156	98.99	326.8	110	41.65	40.39	0.0311
	IL-5	156	0.7553	0.8279	110	0.7062	0.6693	0.5926
	IL-7	156	0.7442	0.6159	110	0.6886	0.4264	0.3829
	LTA	155	0.4188	0.4499	110	0.4174	0.3734	0.9778
	VEGF	155	22.36	82.51	110	14.48	52.62	0.3423
Proinflammatory	**IFNγ**	155	11.93	27.88	110	9.68	14.49	0.3906
	IL-10	155	2.231	5.473	110	1.745	2.900	0.3487
	IL-13	155	3.463	2.437	110	3.255	1.880	0.4305
	IL-1β	155	3.451	12.22	110	1.685	2.486	0.0810
	IL-2	155	3.161	3.922	110	2.815	2.312	0.3664
	IL-4	155	0.6397	0.7944	110	0.5895	0.7576	0.6042
	IL-6	155	3.024	6.367	110	2.660	4.818	0.5951
	IL-8	155	41.5	149.1	110	14.0	53.5	0.0358
	TNF	155	0.7482	1.055	110	0.7787	0.9975	0.8121
Vascular Injury	**CRP**	155	1010	2909	110	547.4	1605	0.0978
	SAA	155	109.1	378.7	110	71.76	53.12	0.2257
	sICAM-1	155	19.87	44.79	110	13.22	41.83	0.2202
	sVCAM-1	155	22.26	22.09	110	22.61	21.50	0.8953

Mean protein levels (pg/mg total protein) with standard deviations (Std Dev) in stool homogenates

N = number of measurements for each subject group

Gray shading indicates significant ($p<0.05$) difference between patients and controls by t-test

Table 6. Comparison of levels of immune and angiogenesis factors in stool from PD patients and their spouses separated by sex

		PATIENT IS FEMALE				PATIENT IS MALE			
Panel	**Factor**	**N**	**Mean Dif (pg/mg)**	**Std Dev**	**p-value**	**N**	**Mean Dif (pg/mg)**	**Std Dev**	**p-value**
Angiogenesis	**Flt1**	10	1.776	3.077	0.1013	29	-0.3635	4.451	0.6635
	PlGF	10	0.6796	0.6393	0.0084	29	-0.2227	0.9099	0.1982
	Tie2	10	67.79	122.3	0.1135	29	2.117	168.1	0.9464
	VEGF-C	10	70.45	102.4	0.0575	29	-34.11	101.9	0.0824
	VEGF-D	10	8.259	9.029	0.0178	29	-3.290	16.87	0.3028
	bFGF	10	0.3046	0.3821	0.0327	29	-0.04650	0.7164	0.7291
Chemokine	**Eotaxin**	10	4.965	5.986	0.0277	29	-0.6213	6.545	0.6132
	Eotaxin-3	10	16.56	16.12	0.0100	29	-7.192	30.08	0.2084
	IP-10	10	0.2133	0.3305	0.0717	29	-0.01270	0.4037	0.8672
	MCP-1	10	0.1473	0.3382	0.2016	29	-0.03700	0.2909	0.4993
	MCP-4	10	1.897	3.314	0.1037	29	-0.7261	2.526	0.1328
	MDC	10	5.371	6.354	0.0255	29	-2.534	8.185	0.1066
	MIP-1α	10	3.501	5.582	0.0786	29	-0.2698	5.443	0.7914
	MIP-1β	10	1.538	2.092	0.0452	29	-0.2347	2.189	0.5683
	TARC	10	0.3986	0.3828	0.0093	29	-0.1299	0.7797	0.3773
Cytokine	**IL-12/23 p40**	10	0.6052	1.692	0.2874	29	0.04700	2.013	0.9007
	IL-15	10	0.3157	0.4257	0.0437	29	-0.01490	0.8733	0.9275
	IL-16	10	14.05	18.52	0.0399	29	-2.711	12.84	0.2652
	IL-17A	10	0.5709	1.475	0.2521	29	-0.02620	1.953	0.9429
	IL-1α	10	12.97	61.82	0.5236	29	165.9	569.2	0.1277
	IL-5	10	0.4791	0.5910	0.0305	29	-0.08360	0.7022	0.5268
	IL-7	10	0.1915	0.6243	0.3573	29	-0.07800	0.6125	0.4986
	LTA	10	0.1685	0.3000	0.1095	29	-0.1451	0.6120	0.2204
	VEGF	10	34.32	90.45	0.2608	29	10.35	61.63	0.3821
Proinflammatory	**IFNγ**	10	7.957	13.69	0.0993	29	5.571	49.55	0.5569
	IL-10	10	0.2590	1.876	0.6726	29	-0.08170	3.761	0.9093
	IL-13	10	0.5898	2.791	0.5208	29	-0.3801	1.872	0.2922
	IL-1β	10	1.973	6.809	0.3833	29	4.587	21.70	0.2731
	IL-2	10	0.7725	2.373	0.3301	29	-1.030	4.445	0.2307
	IL-4	10	0.02340	0.4506	0.8733	29	-0.1984	0.9819	0.2944
	IL-6	10	1.885	16.49	0.7262	29	-1.142	4.731	0.2124
	IL-8	10	4.943	22.29	0.5009	29	62.76	232.9	0.1654
	TNF	10	0.04880	0.5317	0.7782	29	-0.3561	1.385	0.1848
Vascular Injury	**CRP**	10	-671.7	2062	0.3298	29	933.4	4847	0.3173
	SAA	10	3.567	72.14	0.8792	29	-1.229	110.4	0.9534
	sICAM-1	10	1.930	4.425	0.2011	29	9.393	44.03	0.2689
	sVCAM-1	10	-1.153	14.72	0.8099	29	-5.996	36.20	0.3885

Mean difference (patient – control) between analyte levels (pg/mg total protein) with standard deviations

N = number of measurements for each subject group

Gray shading indicates significant ($p<0.05$) difference between patients and household controls by paired t-test

Table 7. Comparison of levels of immune and angiogenesis factors in stool from female and male subjects

		FEMALE			MALE			
Panel	**Factor**	**N**	**Mean (pg/mg)**	**Std Dev**	**N**	**Mean (pg/mg)**	**Std Dev**	**p-value**
Angiogenesis	**Flt1**	107	5.0901	4.0222	161	4.2406	3.6986	0.0284
	PlGF	107	1.6608	1.1189	161	1.2315	0.8097	0.0001
	Tie2	107	198.30	131.10	161	182.60	136.10	0.1220
	VEGF-C	107	143.00	135.50	161	99.8438	96.4049	0.0018
	VEGF-D	107	21.6802	13.0447	161	17.9639	13.2221	0.0062
	bFGF	107	0.7321	0.5274	161	0.6149	0.4966	0.0287
Chemokine	**Eotaxin**	107	9.4240	5.8295	161	7.9875	5.6485	0.0114
	Eotaxin-3	107	27.7597	21.7379	161	20.4151	17.8129	0.0012
	IP-10	107	0.5139	0.4695	161	0.3859	0.3264	0.0038
	MCP-1	107	0.3866	0.6711	161	0.2341	0.2886	0.0021
	MCP-4	107	4.8139	2.8880	161	4.3472	3.2492	0.0459
	MDC	107	13.2871	8.0057	161	10.3520	7.6223	0.0003
	MIP-1α	107	5.0413	3.9243	161	4.4153	3.8451	0.1046
	MIP-1β	107	2.6501	1.9337	161	2.1786	1.9771	0.0026
	TARC	107	0.8597	0.5619	161	0.6837	0.6456	0.0007
Cytokine	**IL-12/23 p40**	107	2.7865	1.7657	161	2.4273	1.6394	0.0404
	IL-15	107	0.8455	0.6138	161	0.7711	0.6401	0.1224
	IL-16	107	16.7467	13.9424	161	15.3370	17.5305	0.0388
	IL-17A	107	2.2419	1.5414	161	2.8424	8.4484	0.3902
	IL-1α	107	57.7486	170.70	161	86.8717	294.80	0.9141
	IL-5	107	0.7594	0.6656	161	0.7188	0.8265	0.0775
	IL-7	107	0.7752	0.5219	161	0.6853	0.5589	0.0451
	LTA	107	0.4315	0.3812	160	0.4093	0.4435	0.1328
	VEGF	107	19.7649	60.4769	160	18.6258	78.3450	0.1243
Proinflammatory	**IFNγ**	107	11.5263	16.5472	160	10.6408	26.8748	0.0423
	IL-10	107	2.5303	6.1913	160	1.6933	3.0456	0.2343
	IL-13	107	3.5374	2.3020	160	3.2691	2.1655	0.2559
	IL-1β	107	2.4474	3.8429	160	2.8968	11.8805	0.0741
	IL-2	107	3.3511	3.6840	160	2.7942	3.0932	0.0226
	IL-4	107	0.6743	0.8559	160	0.5818	0.7221	0.2818
	IL-6	107	3.6996	7.3178	160	2.3197	4.3774	0.0878
	IL-8	107	30.3315	101.30	160	29.8934	130.90	0.0349
	TNF	107	0.8498	1.0823	160	0.7014	0.9918	0.0186
Vascular Injury	**CRP**	107	737.80	2241.90	160	870.90	2599.60	0.8887
	SAA	107	124.40	453.00	160	72.9955	66.9383	0.1262
	sICAM-1	107	22.3580	59.7253	160	13.5926	27.8402	0.0435
	sVCAM-1	107	25.6117	24.1980	160	20.2614	19.8359	0.0670

Mean protein levels (pg/mg total protein) with standard deviations (Std Dev) in stool homogenates

N = number of measurements for each subject group

Gray shading indicates significant ($p<0.05$) difference between females and males by Wilcoxon rank sums test with normal approximation

Table 8. Association between PD status and levels of stool immune and angiogenesis factors when accounting for potential confounders or effect modifiers

Panel	Factor	Estimate	95% Confidence Limits		p-value
Angiogenesis	Flt1	1.087	-0.01908	2.192	0.0541
	PlGF	1.087	-0.01908	2.192	0.0541
	Tie2	-0.4496	-39.62	38.72	0.9820
	VEGF-C	-9.056	-42.74	24.63	0.5968
	VEGF-D	-1.289	-5.128	2.551	0.5091
	bFGF	0.01910	-0.1271	0.1653	0.7971
Chemokine	Eotaxin	0.1777	-1.476	1.831	0.8325
	Eotaxin-3	0.9905	-4.683	6.663	0.7312
	IP-10	0.02551	-0.08694	0.1380	0.6554
	MCP-1	0.01348	-0.1242	0.1511	0.8471
	MCP-4	0.5197	-0.4079	1.447	0.2708
	MDC	-0.7171	-2.992	1.558	0.5353
	MIP-1α	-0.7171	-2.992	1.558	0.5353
	MIP-1β	0.08906	-0.4774	0.6555	0.7570
	TARC	-0.1315	-0.3080	0.04493	0.1433
Cytokine	IL-12/23 p40	0.02485	-0.4662	0.5159	0.9207
	IL-15	0.1547	-0.03270	0.3421	0.1052
	IL-16	1.367	-3.425	6.159	0.5747
	IL-17A	0.6550	-1.319	2.629	0.5139
	IL-1α	87.51	12.94	162.1	0.0216
	IL-5	-0.003209	-0.2306	0.2241	0.9778
	IL-7	-0.004470	-0.1665	0.1576	0.9567
	LTA	0.004860	-0.1218	0.1315	0.9398
	VEGF	19.25	-2.023	40.52	0.0759
Proinflammatory	IFNγ	3.607	-3.413	10.63	0.3124
	IL-10	1.296	-0.04960	2.641	0.0590
	IL-13	0.1148	-0.5554	0.7851	0.7360
	IL-1β	3.015	0.2192	5.810	0.0347
	IL-2	0.6627	-0.3325	1.658	0.1908
	IL-4	0.06967	-0.1617	0.3010	0.5535
	IL-6	1.415	-0.2765	3.106	0.1007
	IL-8	40.28	4.368	76.19	0.0281
	TNF	-0.04665	-0.3570	0.2637	0.7674
Vascular Injury	CRP	1134	411.7	1857	0.0022
	SAA	1.357	-82.79	85.79	0.9747
	sICAM-1	7.169	-5.786	20.12	0.2767
	sVCAM-1	0.4134	-5.982	6.809	0.8988

Gray shading indicates significant ($p<0.05$) association with PD status parameter by multivariate GLM, multiple imputation = 5

Table 9. No significant associations between PD duration and levels of stool immune and angiogenesis factors

Panel	Factor	N	Mean Square	F-value	R-square	p-value
Angiogenesis	Flt1	156	1.818	0.10	0.000639	0.7534
	PlGF	156	0.1890	0.15	0.000946	0.7022
	Tie2	156	2263	0.10	0.000627	0.7556
	VEGF-C	156	25.62	0.00	0.000014	0.9631
	VEGF-D	156	5.096	0.02	0.000155	0.8769
	bFGF	156	6.417E-03	0.02	0.000131	0.8868
Chemokine	Eotaxin	156	13.89	0.36	0.002347	0.5468
	Eotaxin-3	156	66.44	0.18	0.001172	0.6703
	IP-10	156	7.719E-04	0.00	0.000024	0.9516
	MCP-1	156	4.916E-02	0.14	0.000918	0.7064
	MCP-4	156	1.408E-02	0.00	0.000008	0.9728
	MDC	156	4.651	0.06	0.000408	0.8018
	MIP-1α	156	0.6211	0.03	0.000225	0.8520
	MIP-1β	156	3.292E-02	0.01	0.000050	0.9302
	TARC	156	0.2584	0.60	0.003826	0.4416
Cytokine	IL-12/23 p40	156	2.239E-03	0.00	0.000005	0.9789
	IL-15	156	0.1527	0.37	0.002356	0.5460
	IL-16	156	44.46	0.14	0.000931	0.7044
	IL-17A	156	28.20	0.38	0.002470	0.5365
	IL-1α	156	1.828E+04	0.17	0.001097	0.6805
	IL-5	156	0.0000	0.00	0.000000	0.9958
	IL-7	156	2.756E-03	0.01	0.000047	0.9324
	LTA	155	1.138E-02	0.06	0.000363	0.8135
	VEGF	155	2278	0.33	0.002159	0.5646
Proinflammatory	IFNγ	155	70.83	0.09	0.000588	0.7638
	IL-10	155	9.188	0.31	0.001979	0.5813
	IL-13	155	0.1701	0.03	0.000185	0.8662
	IL-1β	155	77.07	0.51	0.003331	0.4742
	IL-2	155	0.4999	0.03	0.000210	0.8576
	IL-4	155	8.010E-02	0.13	0.000819	0.7229
	IL-6	155	7.499	0.18	0.001194	0.6685
	IL-8	155	116.1	0.01	0.000034	0.9427
	TNF	155	0.1160	0.10	0.000672	0.7480
Vascular Injury	CRP	155	2.194E+07	2.62	0.016736	0.1075
	SAA	155	2.036E+03	0.01	0.000092	0.9056
	sICAM-1	155	906.3	0.45	0.002914	0.5033
	sVCAM-1	155	99.06	0.20	0.001310	0.6537

N = number of measurements

No significant ($p<0.05$) association between PD duration (age at time of stool collection minus age of PD onset) and analyte levels by GLM regression

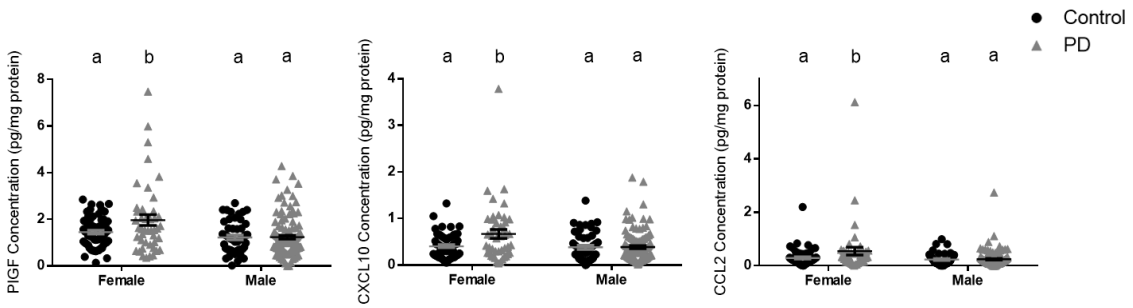

Figure 2. Levels of select stool analytes elevated in female PD patients but not male

Levels of PIGF, CXCL10 (IP-10), and CCL2 (MCP-1) in stool homogenates. Significant ($p<0.05$, Type III SS) interaction between sex and disease status by generalized linear model. Different letters above graphs indicate significant differences (least squares means, $p<0.05$) between groups.

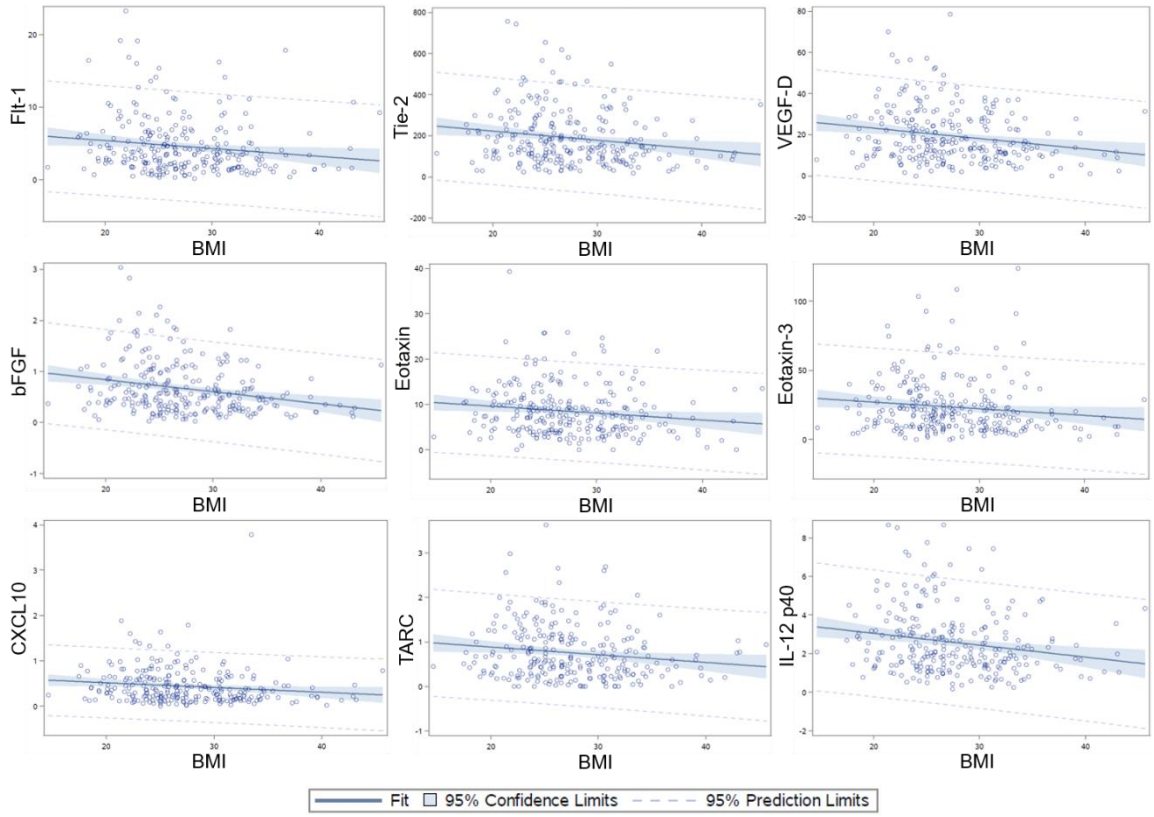

Figure 3. Levels of nine chemokines and angiogenesis factors decrease with increasing BMI

Fit plots for analytes in stool with significant ($p<0.05$) association with body mass index (BMI) by generalized linear model.

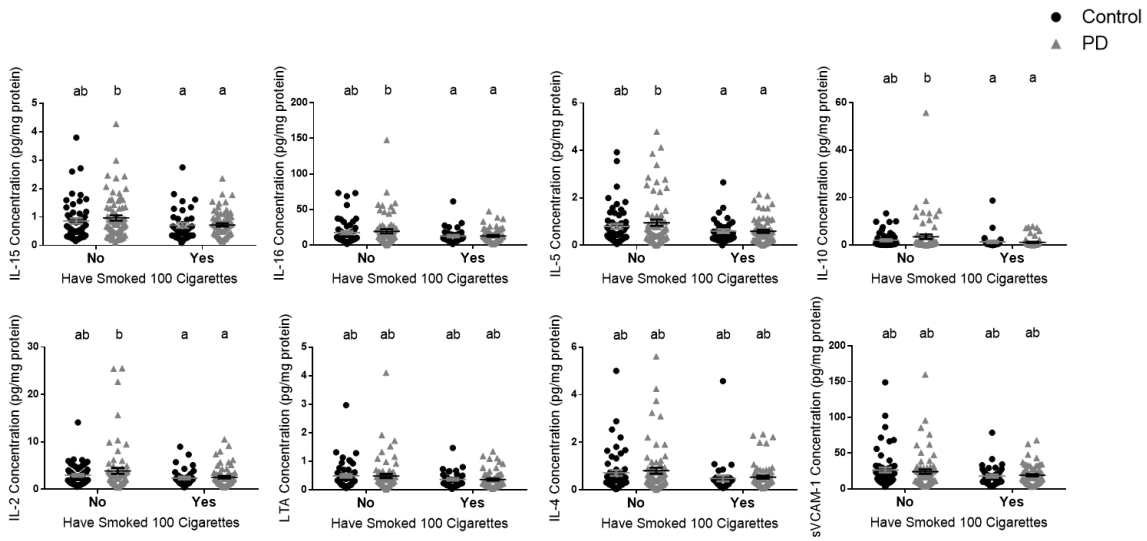

Figure 4. Levels of seven cytokines and one vascular factor are reduced in subjects with a history of smoking

Levels of analytes in stool homogenates with significant ($p<0.05$, Type III SS) effect of smoking by generalized linear model. Different letters above groups indicate significant differences (least squares means, $p<0.05$) between groups.

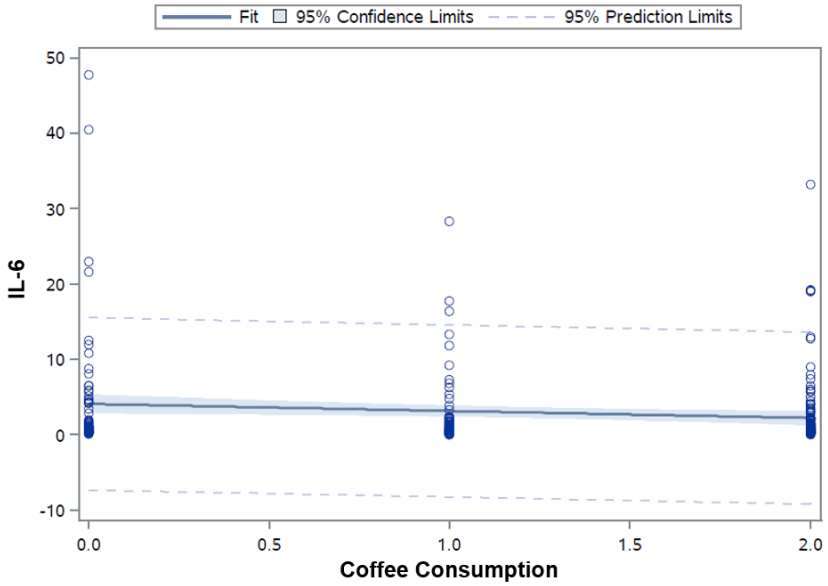

Figure 5. Levels of IL-6 decrease with increasing coffee consumption

Fit plot for levels of IL-6 in stool homogenates. Significant ($p<0.05$) association with coffee consumption by generalized linear model.

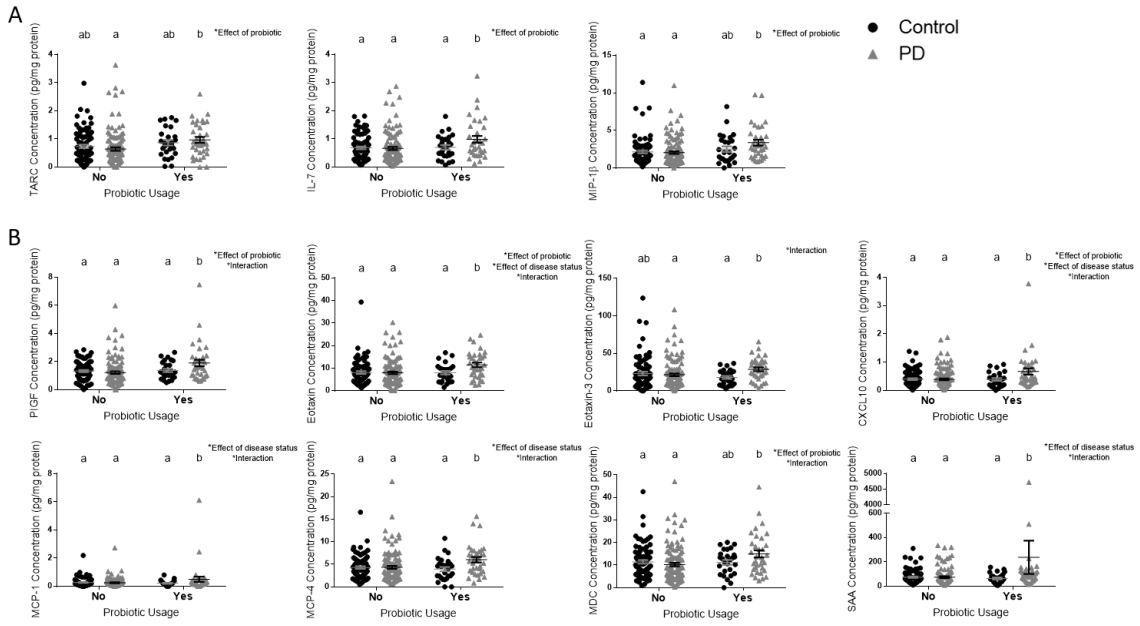

Figure 6. Levels of eleven immune factors elevated in stool of subjects taking probiotics with especially strong effects in Parkinson's disease (PD) patients

Levels of analytes in stool homogenates with significant ($p<0.05$, Type III SS) (**A**) effects of probiotics and/or **(B)** interactions between probiotics and disease status by generalized linear model. Different letters above groups indicate significant differences (least squares means, $p<0.05$) between groups.

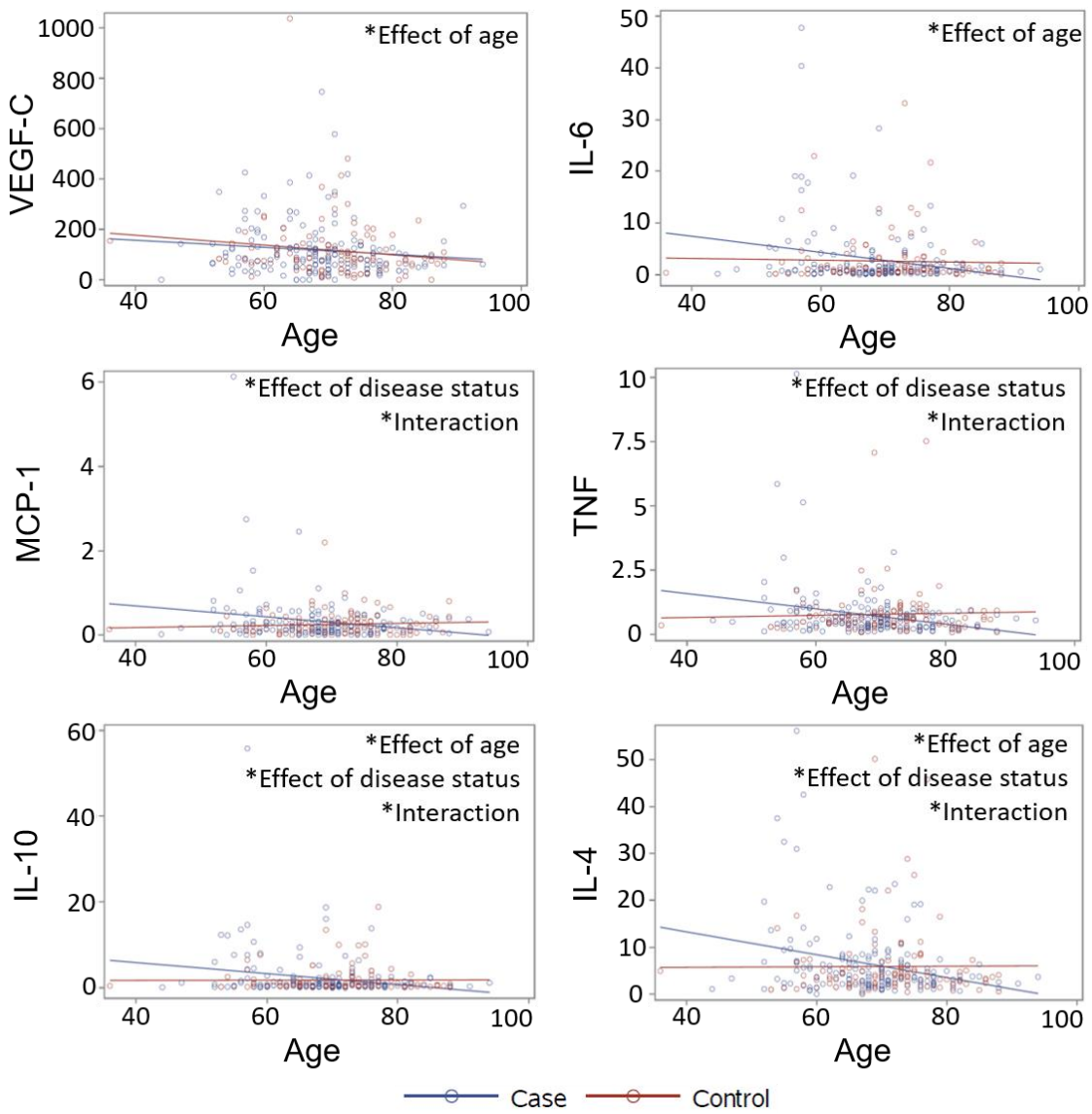

Figure 7. PD patients and controls differ in associations between subject age and levels of stool analytes

Levels (pg/mg) of analytes in stool homogenates from PD patients (blue) and controls (red) as a function of subject age. Significant ($p<0.05$) effects of age, disease status, and interactions between the variables by generalized linear model regression.

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CHAPTER 3: EXPERIMENTAL COLITIS MIMICS INTESTINAL INFLAMMATORY FEATURES OF PARKINSON'S DISEASE AND PROMOTES SUSTAINED T CELL-ASSOCIATED MIDBRAIN NEUROINFLAMMATION AND PARKINSONIAN NEUROPATHOLOGY

Note: The dissertation author independently prepared samples, performed experiments, and collected and analyzed the data reflected in the figures and tables in this chapter with the following exceptions: HPLC was performed by the Emory HPLC Bioanalytical Core; multiplexed immunoassays were performed by the Emory Multiplexed Immunoassay Core; RNA isolation, cDNA preparation, and qPCR from substantia nigra pars compacta tissue was performed in part by a senior research specialist according to the dissertation author's experimental design.

3.1 Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting hundreds of thousands of people in the United States alone (1). Its debilitating motor impairments are caused primarily by the progressive degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) region of the brain. The factors that initiate neuronal dysfunction and lead to neuropathology remain unclear. For years, however, it has been observed that gastrointestinal (GI) dysfunction is associated with PD and that GI symptoms frequently manifest decades before motor symptoms (2). This has prompted theories that pathology in the intestine could promote the development of sporadic PD. Intestinal inflammation has been proposed (3-5) as a key mechanism mediating gut-to-brain PD progression, and evidence that such inflammation is present in PD is accumulating. Indicators of

gut inflammation in PD include documentation of oxidative stress in colon tissue of PD patients (6) and increased levels of the inflammatory indicator calprotectin in PD patients' stool (7). Higher expression of genes encoding proinflammatory cytokines such as TNF, IFNγ, IL-6, IL-1β, CXCL8, and others has been documented in colonic biopsies from PD patients compared to controls (8, 9), and our group recently identified significant elevations in IL-1α, IL-1β, CXCL8, and C-reactive protein (CRP) in stool from PD patients compared to controls (10). We noted that the specific immune mediators identified in these studies were associated with the canonical NFκB signaling pathway, which is persistently activated in chronic inflammatory disorders (11) and appears to be similarly dysregulated in PD (12, 13). Another recent study reported accumulation of cells expressing the lipopolysaccharide (LPS) receptor Toll-like Receptor 4 (TLR4) and cells immunoreactive for the pan T-cell marker CD3 in the colonic mucosa and lamina propria in PD patients (9). Furthermore, epidemiological studies have reported that individuals with inflammatory bowel disease (IBD), a condition characterized predominantly by inflammation of the distal small intestine and/or colon, are at increased risk for development of PD (14-17). Research has also found that treatment with immunosuppressant (18) and specifically anti-TNF (15) therapy significantly reduces IBD-associated PD risk. These findings support the theory that PD pathology could originate in the gut and strongly suggest that gut inflammation is a critical mediator of the epidemiological association between IBD and PD.

The relationship between GI disorders and PD incidence is far from absolute, however. Meta-analysis indicates that the increased risk of PD conferred by IBD is 28-30% (19), and some studies did not identify any increased risk (18) or suggested that the association could be accounted for by other factors such as surveillance bias (16). Two studies have observed an IBD-PD association primarily in men (14, 15), which could simply reflect the greater prevalence of

PD in men (1). Another study reported a stronger effect in women (16), and one other reported no effect of sex on the association (17). There is also known overlap in genetic variants associated with PD and IBD (20, 21), and it has been shown that the disease risk conferred by PD-linked genetic polymorphisms can be impacted synergistically by exposure to environmental risk factors such as pesticides (22). Certainly, PD is a complex and heterogenous disorder which typically necessitates the convergence of multiple risk factors including age, sex, genetic predisposition, and environmental exposures in addition to any contribution from GI pathology.

Our group has investigated the loss of the Regulator of G-Protein Signaling 10 (RGS10) as one such risk factor for inflammation-mediated parkinsonian neuropathology in animal and cell culture models (23-25). RGS10 is highly expressed in central and peripheral myeloid cells where it acts as a negative regulator of inflammatory activity through inhibition of NFκB (23-25). We have shown that RGS10 loss increases susceptibility to immune- and oxidative stress-mediated dopaminergic neurodegeneration in both *ex* and *in vivo* models (23-27). How RGS10 loss might affect PD-relevant gut-brain interactions has not been investigated, but if inflammatory processes play a role, then one would expect these to be heightened or dysregulated in RGS10-deficient animals.

Research testing the theory that gut inflammation contributes to the development of central parkinsonian neurodegeneration is in its infancy. Several studies have now reported that oral administration of the pesticide rotenone induces GI inflammation and alterations in the gut microbiome along with central neurodegeneration and motor deficits (28-32), and one found that these effects were mitigated in TLR4-deficient mice (9), suggesting that inflammatory responses are important mediators of the pathology. One study reported that rats experiencing dextran sodium sulfate (DSS)-induced colitis for seven days exhibited increased microglial activation,

neuroinflammation, and neuron loss in the SNpc and that these effects were mitigated by depletion of peripheral macrophages (33). Male mice given 2.5% DSS for seven days exhibited an increase in proinflammatory IL-1β in the SNpc but no neurodegeneration; a subchronic regimen of 3 weeks of 0.5% DSS was required to elicit loss of DA neurons (34). This group also found that vagotomy prior to induction of colitis mitigated its neurodegenerative effects on the brain (34). These studies are consistent with epidemiological findings linking GI disease and PD in humans and implicate inflammatory processes as well as the vagus nerve in the development of colitis-associated neuropathology. Significant questions remain, however, regarding the protracted effects of GI inflammation on the brain, the mechanisms that mediate them, and the interaction of colitis with sex and genetic and environmental risk factors.

To address these questions, we examined the effects of DSS colitis on male and female WT and RGS10$^{-/-}$ mice weeks after cessation of DSS administration, and we evaluated whether colitis could exacerbate the effects of a known DA neurotoxicant administered at subthreshold doses (**Fig 8**).

3.2 Methods

3.2a Human Subjects

Human colon biopsies from the cohort described by Shannon *et al*. (35) were utilized in this study. PD patients (diagnosed according to United Kingdom Parkinson Disease Research Society Brain Bank criteria) were recruited from the Rush University Medical Center (RUMC) movement disorders clinic after study approval by their Institutional Review Board. Subjects did not have atypical or secondary parkinsonism, and their symptoms were sufficiently mild that they were not being treated with levodopa or dopamine agonists. A healthy control (HC) from

the IRB-approved Tissue Repository from the RUMC Department of Gastroenterology and Nutrition was selected based on age, sex, BMI, and race matching for each PD subject. HCs had no history of gastrointestinal or neurological disease. Exclusion criteria for PD and HC groups were: diabetes, alcohol abuse, use of antiplatelet or anticoagulant drugs, primary gastrointestinal pathology, and any medical, neurological, or psychiatric condition that was not well-controlled.

Subjects underwent unsedated limited unprepped flexible sigmoidoscopy to the distal sigmoid at approximately 20cm from the anal verge. Cold biopsies were obtained using biopsy forceps from visually normal sigmoid colon tissue and were flash-frozen in liquid nitrogen and stored at -80°C until processing.

3.2b Mouse Procedures

$RGS10^{-/-}$ mice generated as described by Lee *et al.* (24) were rederived at Emory University in a new AAALAC-certified specific pathogen-free facility on a mixed C57BL/6J/N background. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC). Baseline genotype comparisons were made using two-month-old male and female $RGS10^{+/+}$ and $RGS10^{-/-}$ littermates. To assess GI motility, mice were fasted two hours, then orally gavaged with a solution of carmine red dye (6%; Sigma-Aldrich) and methylcellulose (0.5%; Sigma-Aldrich) in sterile PBS, placed into individual cages, and observed until they expelled a red fecal pellet. Intestinal transit time was calculated as the time between gavage and appearance of the dye in the feces. At least 48h after *in vivo* experiments were completed, mice were weighed, anesthetized by exposure to isofluorane (Piramal), and cervically dislocated. The colon was dissected, gently pressed and rinsed in PBS to remove feces, and its length measured. Distal colon tissue was frozen in OCT for histology of frozen sections, fixed in 4%

paraformaldehyde (PFA) overnight for whole mount histological preparations, or flash-frozen in liquid nitrogen for molecular assays and stored at -80°C until processing.

To evaluate the impact of colitis on central neuropathology, male and female 2-3 month-old RGS10$^{+/+}$ and RGS10$^{-/-}$ mice were assigned to one of four experimental groups: H2O-Saline, H2O-MPTP, DSS-Saline, or DSS-MPTP (**Fig 8**), with littermates divided across groups. Mice in H2O groups drank autoclaved tap water *ad libitum*. Mice in DSS groups drank autoclaved tap water containing 2% DSS (Affymetrix) *ad libitum* for 5 days after which they were returned to autoclaved tap water. Fifteen days after experiment initiation, mice in MPTP groups received a subacute regimen of 18mg/kg MPTP (Sigma-Aldrich) in saline s.c. (~100uL) each day for 5 days, similar to that reported by Lohr *et al* (36). Mice in Saline groups received 100μL s.c. sterile saline daily. Mice were coded by a researcher unaffiliated with the study so that the researchers conducting the experiments were blinded to genotype and treatment group until data analyses. Mice were maintained on a 12 hour:12 hour light/dark cycle and provided standard chow *ad libitum* throughout the experiment. Staff veterinarians in the Division of Animal Resources (DAR) at Emory University were consulted to ensure humane study endpoints.

Measurements of weight loss, feces consistency, and fecal blood were taken daily during DSS exposure and for 10 days after its removal and scored according to the criteria described in **Table 10**. The sum of the three scores was represented as the daily disease activity index (DAI) for each mouse. Occult blood was detected using Hemoccult II SENSA kits (Beckman Coulter) according to manufacturer's protocol. Approximately six weeks after experiment initiation and one day before experiment termination, ~200μL blood from the facial vein were collected into EDTA-containing microfuge tubes by means of lancet puncture. 100μL were utilized for flow cytometry, and the remainder was centrifuged to separate cells from plasma. Plasma was flash-

frozen in liquid nitrogen and stored at -80°C until processing for multiplexed immunoassay. The following day, mice intended for molecular tissue analysis were decapitated, and the SNpc and bilateral striatum brain regions were rapidly dissected on ice, flash-frozen in liquid nitrogen, and stored at -80°C until processing. Mice intended for brain histology were given an i.p. injection of 40μL Euthasol (Virbac), and when unresponsive, they were perfused with saline until no visible blood remained followed by perfusion with ice-cold 4% PFA in PBS solution for 5min. The brain was then dissected out of the skull and post-fixed in 4% PFA overnight followed by storage in 30% sucrose in PBS solution at least 24 hours before cryosectioning for immunostaining.

3.2c RNA and Protein Isolation

The striata from the left hemisphere were manually homogenized in RIPA buffer (150mM NaCl, 1% Triton X-100, 0.1% SDS, 50mM Tris HCl) with cOmplete Protease Inhibitor cocktail (Roche, 1 tablet/40mL buffer) using an electric pestle. Nigra and colon tissue were homogenized in cold TRIzol reagent (Life Technologies) using a TissueLyser II (Qiagen; 2 cycles, 2min each, 20Hz) and a 5mm stainless steel bead (Qiagen) in order to isolate RNA and protein. RNA was isolated using QIAshredder and RNeasy mini kits (Qiagen) according to the manufacturer's protocol, and concentrations were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher). Protein was isolated from the organic phase of the Trizol preparation by precipitation in methanol and centrifugation followed by resuspension in RIPA buffer with cOmplete Protease Inhibitor cocktail. Protein concentrations were measured using the BCA Protein Assay Kit (Pierce) according to the manufacturer's protocol. RNA was purified with DNAse I (Life Technologies; 0.64μL per 4μg) for 30 min at 37°C, then 10 min at 75°C, and converted to cDNA using SuperScript II Reverse Transcriptase (Life Technologies), dNTPs (Life

Technologies), and random hexamer primers (Integrated DNA Technologies) according to the manufacturer's protocol for the enzyme and as published previously (37).

3.2d Western blot

Protein homogenates (10µg, 20µg for RGS10 measurements) in Laemmli buffer (Bio-Rad) were resolved on 4-20% Mini-PROTEAN TGX Precast gels (Bio-Rad) and transferred to membranes using the Trans-Blot Turbo System (Bio-Rad) according to the manufacturer's protocol. Total protein was visualized using the REVERT Total Protein Stain (LI-COR) according to the manufacturer's protocol and imaged on an Odyssey Fc 2800 (LI-COR). Blots were then cut to facilitate single exposure to multiple antibodies, washed, blocked for ~1h, and incubated overnight with primary antibodies in 5% powdered milk in Tris-buffered saline with 0.1% Tween-20 (TBST). Blots were washed and incubated one hour with appropriate HRP-conjugated secondary antibodies. Antibodies are detailed in **Table 11**. Chemiluminescent signal was imaged on Azure Biosystems C400 system. Bands were quantified by densitometry using Image Studio Lite v5.2. Gut protein levels were normalized to levels of β-actin in each sample from the same blot. β-actin was deemed an inappropriate loading control for striatum samples because its levels differed significantly by experimental group (data not shown), so levels of all targets measured in striata were normalized to total protein.

3.2e Quantitative PCR (qPCR)

qPCR was performed as described previously (38) with minor modifications. For each qPCR reaction, 25ng cDNA was run in triplicate with SYBR Green PCR Master Mix (Life Technologies) and 150nM validated forward and reverse oligonucleotide primers (Integrated

DNA Technologies; **Table 12**) on an ABI Prism 7900 HT Fast Real-time PCR System (Applied Biosystems). Averaged triplicate target gene Ct values were normalized to the averaged values of two housekeeping genes, *RNA18SN5* and cyclophilin (*PPIA*) for human samples and *Gapdh* and *Ppia* for mouse samples. Because Ct values are inversely associated with gene expression, relative mRNA expression was calculated by subtracting normalized Ct values from a standard number (19).

3.2f HPLC

Levels of dopamine and related analytes in the striatum from the right hemisphere were evaluated by HPLC as described previously (39) with the following modifications: Tissue was sonicated (Tissue-Tek, output 3, duty cycle 30%) in 20x volume of 100mM perchloric acid and then transferred to 0.45μm PVDF spin-filter (Grace Davison Discovery Science). Levels of dopamine, DOPAC, HVA, and L-DOPA were quantified by comparing peak areas to standards.

3.2g Multiplexed immunoassays

Distal colon tissue was homogenized in buffer (125mM Tris, 15mM MgCl2, 2.5mM EDTA pH 7.2, 1% Triton X-100, 1 tablet cOmplete protease inhibitors [Roche] per 10mL buffer) using a TissueLyser II (Qiagen; 2 cycles, 2min each, 20Hz) and a 5mm stainless steel bead (Qiagen). Remaining solids were pelleted and supernatants collected. Protein concentrations in supernatants were determined using the BCA Protein Assay Kit (Pierce) according to the manufacturer's protocol and adjusted to 7μg/μL with homogenization buffer.

Colon supernatants and plasma were diluted 1:1 with homogenization buffer immediately before multiplexed immunoassay. Levels of IFNγ, IL-10, IL-12p70, IL-1β, IL-2, IL-4, IL-5, IL-

6, CXCL1, and TNF were measured using the V-PLEX Proinflammatory Panel 1 Mouse Kit (Meso Scale Discovery; MSD) on the MSD QuickPlex instrument and analyzed on the MSD software platform according to the manufacturer's protocol.

3.2h Flow Cytometry

100µL of blood were treated with 2mL of 1X RBC lysis buffer (BioLegend) for 10min at room temperature (RT) in the dark. PBMCs were pelleted, washed with 1x HBSS (Life Technologies), pelleted again, and resuspended in PBS. In a V-bottom plate (Corning), PBMCs were incubated 30min at RT in the dark in 50µL LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies) prepared according to manufacturer's protocol and diluted 1:2000 in PBS. PBMCs were washed in PBS, then incubated 20min on ice in 50µL of solution containing Fc-blocking anti-CD16/CD32 and 9 fluorophore-conjugated antibodies (**Table 11**) in FACS buffer (1mM EDTA and 0.1% sodium azide in PBS). PBMCs were washed in FACS buffer, then incubated 30min on ice with 1% PFA. PBMCs were washed and resuspended in FACS buffer, and 10µL AccuCheck Counting Beads (Thermo Fisher) were added to each sample before evaluation by LSR II flow cytometer and FACSDiva software (BD Biosciences). Results were analyzed using FlowJo 10.4.2 according to the gating strategy in **Figure 9**. Frequencies and counts of each cell population and geometric MFIs of markers were assessed. Cell counts were calculated according to the manufacturer's protocol for 100µL blood.

3.2i Immunostaining

For staining frozen gut sections, 7µm sections of rolled distal colon tissue from $RGS10^{+/+}$ and $RGS10^{-/-}$ mice were cut from frozen OCT blocks and mounted on slides, then fixed in 4%

PFA for 10min prior to staining. Tissue for gut whole mounts was prepared by dissecting the muscle and myenteric plexus layer of fixed distal colon tissue from the rest of the tissue containing the lamina propria with the aid of a dissecting microscope. 40μm sections of SNpc were cut on a freezing microtome from fixed brains and rinsed in PBS.

Brain tissue intended for brightfield microscopy was incubated in 3% hydrogen peroxide for 15min at 37°C to block endogenous peroxidase activity. All tissue was blocked 1h at RT in PBS with 5% Normal Donkey or Goat Serum (NDS, NGS; Jackson ImmunoResearch) depending on the host of the secondary antibody used, then incubated in primary antibodies diluted in PBS with 1% NDS/NGS for 1h at RT for frozen sections and overnight at 4°C for whole mounts and brain sections. Tissue was rinsed thoroughly with PBS and then incubated for 1h at RT with secondary antibodies diluted in PBS with 1% NDS/NGS. For gut tissue, 0.3% Triton X-100 (EK Industries) was included in blocking, and primary and secondary antibody incubations. Biotinylated secondary antibodies were used for brain immunostaining; after this incubation, sections were washed in PBS and transferred to avidin-biotin-peroxidase complex (ABC) (Vector Laboratories) solution for 1h at 4°C, then developed using DAB Tablets (Sigma-Aldrich) according to the manufacturers' protocols. Antibody details are listed in **Table 11**, with more concentrated antibody dilutions used for whole mounts or brain sections and less concentrated for frozen gut sections.

After washing with PBS, free-floating tissue was transferred to microscopy slides. Tissue for immunofluorescence was treated with VECTASHIELD mounting medium containing DAPI (Vector Laboratories). Images were obtained on a Nikon Eclipse 90i microscope with a Nikon DS-Qi1MC camera and Nikon NIS Elements software.

3.2j Statistics

Paired, two-tailed t-test was used to compare matched HC and PD colonic biopsy data. Two-way repeated measures ANOVA with Sidak's *post hoc* was used to compare littermate-paired samples and DAI of $RGS10^{+/+}$ and $RGS10^{-/-}$ mice over the course of DSS colitis. Two-way ANOVA with Tukey's *post hoc* was used for all other genotype and treatment comparisons. Male and female mouse data were evaluated separately. Pearson correlations were used to assess associations between variables. These statistical analyses were performed using GraphPad Prism 6, and $p<0.05$ was considered significant. Data are represented as mean ±SEM unless otherwise specified.

Supervised machine learning using a random forest ensemble was employed to rank the various measures in this study according to the value of their contribution to a regression model of striatal tyrosine hydroxylase (TH) protein levels. 34 variables were included in the model. Data on phosphorylated TH levels and nigral *Th* mRNA abundance were excluded as they were considered too closely related to striatal TH protein levels to be informative. HPLC measures of dopamine and its metabolites were excluded because levels of these molecules are directly regulated by TH, and data on striatal DAT and VMAT2 protein levels were excluded because they are known to be co-regulated with TH. Separate models were built for male and female mice with 55 individuals included for each. Due to this relatively small sample size, the data were not partitioned, and models were trained on the entire dataset in order to generate descriptive information for this study. Three hundred decision trees were built, and nine variables were considered for partitioning in each tree. The final models were high-quality representations of the dataset (**Figure 10**). These models were developed in R using RStudio (40) and the graphic user interface rattle (41) with packages "rattle" (41) and "magrittr" (42).

3.2k Study Approval

The aspects of this study involving human tissue were conducted in accordance with the principles of the Declaration of Helsinki and were approved by the Rush University Medical Center Institutional Review Board and registered at Clinicaltrials.gov (NCT01155492). All subjects provided written informed consent. The aspects of this study involving animals were conducted in accordance with National Institutes of Health animal care and use policies and were approved by the Emory University Institutional Animal Care and Use Committee.

3.3 Results

3.3a RGS10 deficiency induces intestinal inflammation and dysfunction

This study utilized $RGS10^{-/-}$ mice to evaluate the effects of colitis in animals with increased sensitivity to inflammation-mediated neuropathology. Untreated $RGS10^{+/+}$ and $RGS10^{-/-}$ mice were compared to determine baseline differences in the intestinal environment. RGS10-expressing cells were present in the murine colon and appeared to be primarily of myeloid rather than lymphocyte or neuronal lineage (**Fig 11A, Fig 12)**. Evidence of inflammation in the colon of $RGS10^{-/-}$ mice was also observed. While there was no significant difference in body weight between $RGS10^{+/+}$ and $RSG10^{-/-}$ mice, the colon lengths of $RGS10^{-/-}$ mice normalized to body weight were shorter than their WT littermates (**Fig 11B**), and levels of the proinflammatory cytokine IFNγ were significantly increased in $RGS10^{-/-}$ colon tissue (**Fig 11C**). No significant differences were found in levels of IL-10, IL-12 p70, IL-1β, IL-2, IL-4, IL-5, IL-6, CXCL1, or TNF (data not shown). Expression of the *Chat* gene encoding choline acetyltransferase, the enzyme which catalyzes the production of the neurotransmitter

acetylcholine, was significantly reduced in RGS10$^{-/-}$ colon tissue in both males and females (**Fig 11D**), while intestinal transit time was significantly increased in RGS10$^{-/-}$ males only (**Fig 11E**). Regardless of sex, when RGS10$^{-/-}$ mice were exposed to the colitic agent DSS, they developed more severe and more persistent colitis than WT mice (**Fig 11F**).

3.3b Inflammatory features of DSS colitis are consistent with those observed in colon tissue from Parkinson's disease patients

DSS colitis is commonly used to model aspects of IBD (43), but to determine whether its effects are relevant to or resemble features that occur in PD, we measured indicators of inflammation in sigmoid colon biopsy tissue from PD patients and matched healthy controls (HCs) and measured the murine equivalents in distal colon tissue from RGS10$^{+/+}$ and RGS10$^{-/-}$ mice. We observed a marked increase in NFκB p65 protein levels in the colon of PD patients compared to HCs (**Fig 13A**). Consistent with our previous findings in mice regarding RGS10 and NFκB (24, 25), NFκB p65 levels were also significantly increased in the colon of RGS10$^{-/-}$ mice (**Fig 13B**). In PD colon tissue, expression of *LCN2* – a common indicator of gut inflammation – *NOS2* – an indicator of oxidative stress – *PTPRC* which encodes CD45 – a marker expressed on immune cells – and *CD8B* – a marker of cytotoxic T cells – was higher than in HCs. Expression of *SNCA* which encodes α-synuclein trended higher but did not differ statistically from HCs (**Fig 13C**). DSS affected expression of these same factors in mice, producing significantly higher levels of each in the colon tissue of RGS10$^{+/+}$ and RGS10$^{-/-}$ male mice and of *Lcn2* and *Nos2* in females of both genotypes. Expression of *Cd8b* and *Snca* increased with DSS only in RGS10$^{-/-}$ and not RGS10$^{+/+}$ females. A significant effect of genotype

on *Lcn2* and *Ptprc* expression was also observed for male mice, with higher levels present in RGS10$^{-/-}$ colon tissue (**Fig 13D**), reflecting the greater severity of colitis in RGS10-null animals.

3.3c Colitis and RGS10 deficiency perturb nigrostriatal dopaminergic systems and augment effects of MPTP

To evaluate how colitis might impact the brain and how it might interact with other factors such as sex, genetic predisposition to excessive inflammatory responses, and neurotoxicants, we exposed male and female RGS10$^{+/+}$ and RGS10$^{-/-}$ mice to DSS in their drinking water for five days, returned them to standard water for 10 days to allow colitis to resolve, then dosed some of the mice with a subacute regimen of the known nigrostriatal neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Five weeks after the last exposure to DSS and three weeks after the last exposure to MPTP, SNpc and striatum of the mice in four experimental groups (H2O-Saline, H2O-MPTP, DSS-Saline, DSS-MPTP) were evaluated for indications of neuropathology (**Fig 8)**.

Levels of mRNA encoding tyrosine hydroxylase (TH) – the enzyme which catalyzes the rate-limiting step in the synthesis of dopamine – in the SNpc were significantly reduced in the RGS10$^{-/-}$ DSS-MPTP group compared to both genotypes in the male H2O-Saline and H2O-MPTP groups and compared to female RGS10$^{+/+}$ and RGS10$^{-/-}$ H2O-Saline and RGS10$^{+/+}$ DSS-Saline mice (**Fig 14A**), indicating that the combination of colitis, neurotoxic insult, and genetic susceptibility were necessary to produce significant reductions in *Th* expression. While nigral *Th* expression measured by qPCR correlated significantly with striatal TH protein measured by western blot (**Fig 15A**), protein levels of TH in the striatum were more sensitive to experimental manipulations. They were significantly reduced compared to RGS10$^{+/+}$ H2O-Saline levels by

MPTP in males and females as well as by colitis alone in male mice (**Fig 14B**). A similar pattern was observed in the abundance of TH protein in the SNpc by immunostaining (**Fig 14C**). A significant effect of genotype on striatal TH levels was also observed, with $RGS10^{-/-}$ mice exhibiting lower striatal TH levels compared to $RGS10^{+/+}$ mice in all experimental groups. The lowest levels of striatal TH were measured in $RGS10^{-/-}$ DSS-MPTP males, and TH in this group was significantly reduced compared to males given DSS or MPTP alone (**Fig 14B**).

Levels of dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT2), which regulate dopamine signaling along with TH, were measured and found to correlate with each other and with TH (**Fig 15B**). Patterns in DAT levels in the striatum mirrored those of TH with the exception that colitis prior to MPTP did not produce significantly greater reductions in striatal DAT than did MPTP treatment alone (**Fig 14D**). Striatal VMAT2 levels typically do not diminish markedly unless substantial degeneration of nerve terminals has occurred (44), and we observed significant reductions in VMAT2 only in $RGS10^{-/-}$ male H2O-MPTP and DSS-MPTP mice compared to $RGS10^{+/+}$ H2O-Saline mice (**Fig 14E**). These data suggest that, while colitis does impact the health of DA neurons in males, it is not sufficient to induce pronounced neurodegeneration.

3.3d Increased activity of TH following colitis prevents dopamine deficiency

A lack of colitis-induced neurodegeneration is supported by measurements of active TH as well as dopamine (DA) and its metabolites DOPAC and HVA. Phosphorylation of TH at serine 40 (pSer40) enhances the enzyme's activity (45), and the ratio of TH-pSer40 to total TH indicates the proportion of the enzyme that is in this active state. Both genotype and treatment affected striatal TH-pSer40:TH ratios in male and female mice in this study. $RGS10^{-/-}$ mice

exhibited increased values compared to RGS10$^{+/+}$ mice. Significant increases relative to RGS10$^{+/+}$ H2O-Saline controls were found in DSS-Saline striata of both genotypes in males and in RGS10$^{-/-}$ DSS-Saline striata in females relative to RGS10$^{+/+}$ DSS-MPTP (**Fig 16A**).

Accordingly, while MPTP-treated mice of both sexes and genotypes exhibited significant reductions in striatal DA compared to saline-dosed control mice (**Fig 16B**), DSS treatment did not significantly impact levels of DA or its metabolites (**Fig 16B, 16C**). MPTP had a less pronounced effect on levels of DOPAC and HVA, only producing slight and inconsistent reductions compared to saline-treated groups (**Fig 16C**). Under neurodegenerative conditions, the ratio of levels of DOPAC and HVA to DA can increase as inadequate DA supplies are more rapidly metabolized. While we identified a significant main effect of treatment on the ratio of DA metabolites to DA, differences among groups by *post hoc* tests were minimal (**Fig 16D**). In female mice, we found a significant main effect of genotype on levels of DA and HVA with slightly lower levels in RGS10$^{-/-}$ mice (**Fig 16B, 16C**). Levels of L-DOPA, the precursor to DA, were also measured by HPLC but were so low as to be undetectable in many samples, and no differences among experimental groups were apparent (data not shown).

3.3e RGS10 deficiency impacts peripheral blood immune cell populations

To assess whether modulation of dopaminergic activity in the brain weeks after colitis might be the result of persistent systemic inflammation, peripheral blood mononuclear cells (PBMCs) were analyzed by flow cytometry at the endpoint of the experiment (**Fig 9**). RGS10$^{-/-}$ DSS-treated males and females as well as MPTP-treated females had more Ly-6C^{-} MHC-II^{+} non-classical monocytes in circulation compared to RGS10$^{+/+}$ both as a proportion of total immune cells and by absolute counts (**Fig 17A**). The functionality of these cells also appeared to

be impacted by RGS10 deficiency. In $RGS10^{-/-}$ DSS-treated males and in females in all experimental groups, this population of monocytes had many cells which expressed reduced levels of CD11b compared to $RGS10^{+/+}$ mice (**Fig 17A**).

Significant genotype effects were also observed for T cells. In males, $RGS10^{-/-}$ mice in H2O-Saline, DSS-Saline, and DSS-MPTP treatment groups had reduced counts of $CD4^+$ T cells compared to $RGS10^{+/+}$ H2O-Saline controls. The frequencies of these cells were reduced in the same experimental groups in females, but the only significant difference in counts was between $RGS10^{+/+}$ H2O-Saline and $RSG10^{-/-}$ DSS-MPTP females. $CD4^+$ T cell frequencies and counts in $RSG10^{-/-}$ DSS-Saline male mice were reduced compared to their $RGS10^{+/+}$ counterparts in the same experimental group (**Fig 17B**). Female mice exhibited no differences in peripheral blood $CD8^+$ T cell populations, but a significant main effect of genotype (two-way ANOVA, $p<0.05$) was found for males, and male $RGS10^{-/-}$ DSS-Saline mice had reduced frequencies and counts of these cells compared to H2O-Saline and H2O-MPTP $RGS10^{+/+}$ mice (**Fig 17B**).

The aforementioned alterations in PBMC populations did not correspond to pronounced peripheral inflammation. No significant differences were observed in levels of IL-10, IL-12 p70, IL-1β, IL-2, IL-4, IL-5, IL-6, or CXCL1 in plasma at endpoint (data not shown). The only differences found were a slight increase in IFNγ in female $RGS10^{+/+}$ H2O-MPTP mice with no effect in males and a decrease in TNF in male $RGS10^{-/-}$ DSS-Saline mice compared to $RGS10^{+/+}$ H2O-Saline mice with no effect in females (**Fig 18**).

3.3f Colitis and RGS10 deficiency promote sustained $CD8^+$ T cell-associated inflammation in the brain

To determine if there were indications that colitis-associated immune responses persisted in the brain weeks after DSS exposure, we evaluated mRNA levels of various immune-related markers in the SNpc by qPCR. We found no differences in the abundances of microglia/myeloid-related mRNA transcripts including *H2-Ab, Il1b, Tnf, Il6, and Tlr4* (data not shown). Furthermore, no significant differences were observed in striatal Iba1 protein levels (**Fig 19**). We also found no differences in astrocyte-associated *Gfap*, in *Nos2*, or in *Snca* (data not shown).

Effects on *Cd4* expression were minimal, with a significant reduction in male $RGS10^{+/+}$ DSS-Saline compared to $RGS10^{+/+}$ H2O-MPTP groups and no significant effects in females (**Fig 20A**). Significant main effects of genotype and treatment were observed for *Cd8b* expression in males, however, and $RGS10^{-/-}$ H2O-MPTP and DSS-treated mice of both genotypes had significantly higher levels of *Cd8b* mRNA compared to $RGS10^{+/+}$ H2O-Saline mice. The highest levels were observed in $RGS10^{-/-}$ DSS-MPTP males (**Fig 20B**). A main treatment effect on *Cd8b* was also found in females, but its expression was only significantly increased in $RGS10^{-/-}$ DSS-MPTP females compared to $RGS10^{+/+}$ H2O-Saline controls (**Fig 20B**). Expression of *Ifng*, which encodes a key cytokine produced by CD8+ T cells and which correlated with *Cd8b* mRNA levels (**Fig 20D**), was also higher in the SNpc in male $RGS10^{-/-}$ H2O-MPTP and DSS-treated mice of both genotypes compared to $RGS10^{+/+}$ H2O-Saline mice (**Fig 20C**). Treatment and genotype effects on *Ifng* expression were observed in females, with DSS treatment increasing expression compared to $RGS10^{+/+}$ H2O-Saline and H2O-MPTP animals (**Fig 20C**).

3.3g $CD8^+$ T cell-associated inflammation in the SNpc is associated with striatal TH levels in males but not females

We utilized a random forest ensemble algorithm to rank the central and peripheral immune measures obtained in this study according to the closeness of their association with striatal TH levels (**Fig 21**). Separate random forests were built for males and females, and the final models explained 38.52% of variance for males and 38.23% for females. For both sexes, MPTP had the strongest impact on levels of striatal TH. In males, *Cd8b* and then *Ifng* expression in the SNpc were the next factors most highly associated with TH followed by colitis DAI score, *Tnf* in the SNpc, and TNF in plasma. After MPTP in females, the most highly ranked factors were counts, frequencies, and the expression of CD11b on MHC-II+ monocytes in the blood followed by *Il1b* expression in the SNpc, genotype with respect to RGS10, and *Snca* in the SNpc. Of note, nigral *Cd8b* expression was not found to be useful in building a regression model for striatal TH in females, and colitis score minimally so. Factors that were found to have no association with TH levels in both sexes included concentrations of IL-1β and IL-6 in plasma, the ratio of $CD4^+$ to $CD8^+$ T cells in blood, and, interestingly, *Cd4* expression in the SNpc (**Fig 21**).

3.4 Discussion

Since intestinal inflammation has been identified in subjects with sporadic PD and has been reported to increase risk for the development of this disorder, this study evaluated lasting neurological and neuroinflammatory effects of colitis and examined their interactions with other factors including sex, genetic susceptibility to hyperinflammatory responses, and exposure to another neurological insult.

While the GTPase-activating protein RGS10 has been previously identified as an anti-inflammatory regulator in the periphery and the CNS (23-25) this study is the first to describe its

role in the intestine. Prior research from our group demonstrated that peripheral myeloid cells express high levels of RGS10 (25), and others have reported minimal RGS10 expression in gut T cells (46). Consistent with these findings, we identified RGS10 expression in the mouse colon primarily in cells that express the monocyte/macrophage marker CD68. RGS10-null immune cells have also been shown to exhibit heightened pro-inflammatory responses (24, 25), and we observed indicators of intestinal inflammation in RGS10$^{-/-}$ mice in this study. RGS10$^{-/-}$ mice had shorter colons – a common indicator of inflammation – than their WT littermates, and higher levels of the transcription factor NFκB p65 and the proinflammatory cytokine IFNγ were present in RGS10$^{-/-}$ colon tissue. While macrophages can produce IFNγ under certain conditions (47), other cell types have been identified as primary contributors to IFNγ responses (48), so the increased levels of this cytokine that we measured may reflect the effects of RGS10-deficient macrophages on T cells and possibly NK cells in the intestinal environment.

RGS10 KO also resulted in reduced expression of choline acetyltransferase (*Chat*), which is necessary for production of the neurotransmitter acetylcholine. *Chat* is expressed by enteric neurons but also by colonic epithelial cells (49) and some immune cells (50). Given the relative frequency of intestinal cell types in colon tissue, it is likely that the measured *Chat* levels primarily reflect those in neurons and epithelial cells rather than immune cells. Levels of intestinal *Chat* and acetylcholine are modulated by inflammation and are suppressed in infection (51), experimental colitis (52), and by incubation with individual proinflammatory cytokines (53). Thus, enhanced inflammatory activity of RGS10$^{-/-}$ immune cells may be responsible for inhibition of acetylcholine synthesis. This deficit could further potentiate RGS10 deficiency-associated inflammation, as acetylcholine is a primary contributor to the cholinergic anti-

inflammatory pathway, which limits the proinflammatory activity of intestinal immune cells (54), particularly macrophages, in which it inhibits activation of NFκB (55, 56).

Acetylcholine also stimulates smooth muscle activity, thereby promoting GI motility. Reduced levels of acetylcholine would be expected to impair colonic motility, and indeed, RGS10$^{-/-}$ male mice exhibited longer intestinal transit times compared to WT littermates, although this was not consistently true for females. Treatment with cholinergic agonists like acetylcholine has also been shown to reduce the severity of experimental colitis (56, 57) and to promote intestinal epithelial cell proliferation (58). With reduced colonic acetylcholine production and low-grade baseline colonic inflammation, RGS10$^{-/-}$ mice developed more severe DSS colitis that resolved more slowly than in WT mice.

Regardless of genotype, male mice exposed to DSS displayed increased expression of inflammation-related genes *Lcn2* and *Nos2*, immune cell-related genes *Ptprc* and *Cd8b*, as well as *Snca* which encodes a key protein associated with PD pathology and which is upregulated in the intestine under inflammatory conditions (59). The effects of DSS were somewhat blunted in females, with no significant difference in *Ptprc* and DSS-induced increases in *Cd8b* and *Snca* only in RGS10$^{-/-}$ mice. The sex differences observed in severity of colitis are consistent with previous reports (60) and may be one factor that contributed to limited observation of colitis-related neuropathology in females in our study.

Importantly, we observed that expression of *LCN2*, *NOS2*, *PTPRC*, and *CD8B* was significantly elevated in colon tissue from PD patients compared to healthy matched controls, with a similar trend in *SNCA* expression. These findings provide further evidence that intestinal inflammation is present with remarkable consistency in the GI tract of individuals with PD and demonstrate that many of the same immunological processes are similarly activated in PD and

experimental colitis. This also supports the epidemiological association between IBD and PD (14-17).

The markedly increased levels of NFκB p65 that we observed in colon tissue from PD patients further confirms ongoing inflammatory activity in the gut in PD. It is also consistent with findings of increased levels of specific proinflammatory cytokines and chemokines regulated by NFκB in colon biopsies (8) and stool (10) from PD patients, with reports of increased fecal calprotectin (7) which can be stimulated through NFκB-associated pathways (61), and with reports of enhanced TLR4 signaling (9) which induces nuclear localization of NFκB.

In PD patients, hallmarks of neurological impairment include reductions in levels of TH (62) and DAT (63), and these pathological changes can be observed well before the onset of motor symptoms (64). Reductions in *TH* mRNA in the SNpc have also been reported in PD, and this is considered a consequence of neurodegeneration (65). Administration of the neurotoxicant MPTP in rodents has been developed as a model that recapitulates many of these observations, producing striatal TH and DAT deficits as well as reductions in VMAT2 which are associated with neurodegenerative terminal loss (66). PD is more common in men than women (67), and the effects of MPTP are typically reported to be more severe in male mice than female (68, 69). The subacute regimen of MPTP utilized in this study produced the anticipated results, significantly reducing TH and DAT levels in the striatum without producing severe neuron damage that may have rendered any augmentation of the phenotype undetectable. RGS10 deficiency did exacerbate the effects of MPTP in both sexes, and males were most susceptible; significant reductions in VMAT2 were only observed in RGS10$^{-/-}$ males. Interestingly, levels of striatal VMAT2 appeared to be higher in females than in males in this study. Increased VMAT2 function in females has been reported previously (70), and higher expression of VMAT2 confers

protection against MPTP-mediated neuron loss (36). It is possible that this also contributes to the resistance of females to colitis-associated neuropathology.

A key finding in this study is that colitis induced pathological changes in DA neuron function in males that were still apparent five weeks after exposure to DSS. Levels of TH and DAT in the striatum were significantly reduced in DSS-treated males compared to H2O-Saline controls. These deficiencies were not as extreme as those produced by MPTP, but they were statistically comparable. As DSS does not cross the blood-brain barrier, these effects are mediated by its activity in the periphery (71). RGS10 deficiency also had a subtle negative impact on dopaminergic neuron health, with TH, DAT, and VMAT2 abundance slightly lower in $RGS10^{-/-}$ mice compared to their $RGS10^{+/+}$ counterparts. The effects of colitis appeared to layer with those of MPTP and RGS10 deficiency, and the striatal TH levels in male $RGS10^{-/-}$ DSS-MPTP mice were significantly lower than in $RGS10^{+/+}$ or $RGS10^{-/-}$ mice treated with DSS or MPTP alone. Additionally, *Th* gene expression was significantly reduced in the SNpc only in $RGS10^{-/-}$ DSS-MPTP mice, though nigral TH protein levels appeared to reflect the patterns observed in striatal TH. These findings are in agreement with other studies demonstrating that DSS colitis can impair TH+ neurons (33, 34), and, importantly, they reveal that these effects persist long after the apparent resolution of gastrointestinal symptoms. They also support epidemiological studies suggesting that IBD increases the risk for developing PD. Enhanced pathology in the absence of RGS10, an anti-inflammatory regulator, reaffirms the pathologic potential of neuroinflammation in the system. While no genetic association between RGS10 and PD has been reported, it is noteworthy that myeloid cells downregulate RGS10 expression in response to inflammatory stimuli (23); therefore chronic gut inflammation may induce

deficiency in RGS10 in the intestine which would exacerbate inflammatory conditions in a feed-forward cycle.

Importantly, we found that colitis alone did not induce significant reductions in VMAT2 in dopaminergic terminals and did not appear sufficient to produce a neurodegenerative phenotype with dopamine deficiency. While levels of striatal TH protein decreased in DSS-treated mice, the activity of this enzyme – reflected by the ratio of TH protein phosphorylated at Ser40 to total TH protein – increased in RGS10$^{-/-}$ females and in males of both genotypes. This effect likely accounts for the fact that there was no decline in levels of DA or its metabolites measured by HPLC. Increased activity of TH has also been reported in the brains of PD patients *post mortem* (62). This compensatory mechanism is not entirely without risk, however. N-terminal phosphorylation of TH which increases the enzyme's activity also promotes its ubiquitination and degradation (72). Thus, this modulation can sustain DA levels in the short-term but with time can exacerbate TH protein deficits, contributing to progressive neurological degeneration such as is observed in PD (73). Together, these findings indicate that, while acute colitis may be insufficient to produce substantial neurodegeneration on the time-scale evaluated in this study, its effects may increase the risk for neuron loss over time and/or sensitize an individual to additional neurological insults. This aligns with our understanding of sporadic PD as a disorder that typically develops in advanced age and is likely to result from the accumulation of multiple risk factors and gene-environment interactions, including intestinal inflammation.

In an effort to identify potential mechanisms by which DSS-associated inflammation could affect the brain, we collected systemic and central immune-related measures and utilized a supervised machine learning algorithm to identify which factors had the strongest associations with levels of striatal TH, a sensitive indicator of neuropathology. In peripheral immune cell

populations, we observed several strong genotype effects and augmentation of them in DSS-treated animals. RGS10$^{-/-}$ mice had fewer CD4^{+} and CD8^{+} T cells, decreased CD4:CD8 T cell ratios, more MHC-II-expressing Ly-6C- non-classical monocytes, and lower CD11b expression on them. While this study detected minimal functional impact of these differences with regard to plasma concentrations of common cytokines and chemokines, these findings are intriguing because reduced CD4^{+} and CD8^{+} T cells and decreased CD4:CD8 T cell ratios have been reported in peripheral blood of PD patients and found to correlate with the severity of disease (74, 75). Furthermore, a common genetic variant in the human leukocyte antigen (HLA) locus which is associated with increased risk for sporadic PD increases baseline and stimulated expression of MHC-II on peripheral blood monocytes (22, 76).

Our random forest algorithm indicated that in females, measurements related to MHC-II^{+} monocytes in the blood were the variables most closely linked to TH. While this population constitutes only a small percentage of the immune cells in blood, the participation of MHC-II-expressing peripheral myeloid cells in neuropathology has been documented. Infiltration of peripheral monocytes into the CNS has been observed in animal models of neurological damage and degeneration, and these cells are reported to express persistently high levels of MHC-II, to exhibit a proinflammatory phenotype, and to contribute to neurodegeneration (77, 78). Despite the lack of significant differences found among experimental groups in many myeloid-related immune markers in the brain in this study, our model suggests that peripheral MHC-II^{+} cells are key influencers of DA neuron health at least in females. In addition to the peripheral immune cell measurements, nigral expression of *Il1b*, a gene which encodes a proinflammatory cytokine primarily produced by myeloid cells, as well as the presence or absence of RGS10, a protein

known to modulate the inflammatory activity of myeloid cells, were factors most closely associated with TH levels in females.

Our random forest-based model confirms experimental findings throughout this study that colitis exerted significant impacts on TH^+ DA neurons in males but not females. This is in keeping with the epidemiological studies that found associations between IBD and PD in men but not women (14, 15), and it suggests that this could well be due to true sex differences in the gut-brain interactions that occur with intestinal inflammation. The machine learning model also demonstrates that the severity of colitis as indicated by the disease activity index score is a substantially better predicter of striatal TH levels than is the binary classification of DSS or H2O treatment. It was also interesting to note that, while no significant differences in nigral *Tnf* expression were found among experimental groups, this measure was one of the most highly ranked in the random forest model for males, as was the concentration of TNF in plasma. Anti-TNF therapy has been reported to significantly reduce the risk for PD development that is otherwise associated with inflammatory bowel disease (15), and our experimental findings here as well as other studies (23, 79, 80) support the potential of TNF to promote dopaminergic neuropathology.

Intriguingly, this study identified significant increases in *Cd8b* and *Ifng* expression in the SNpc of DSS-treated males, and in males, these variables were ranked as the most highly associated with striatal TH levels after MPTP in the random forest model. Both $CD4^+$ and $CD8^+$ T cells have been found in the brains of PD patients *post mortem* (81), and the possibility that they may contribute to disease pathology is now the subject of active investigation (82). Peripheral blood $CD8^+$ T cells from PD patients have been found to exhibit more indicators of activation and fewer indicators of age-related senescence compared to controls (83), and it has

been demonstrated that exposure to α-synuclein peptides could stimulate IFNγ production in $CD8^+$ T cells from PD patients (84). These exciting findings suggest that $CD8^+$ T cells are likely to be involved in ongoing neuroinflammatory immune responses in PD, and, given their cytotoxic activity, they may be perfectly poised to contribute to nigrostriatal neurodegeneration.

Infiltration of $CD8^+$ T cells into the CNS has also been observed in MPTP mouse and non-human primate models, but there are conflicting reports about whether these cells mediate MPTP-associated neuropathology (81, 85, 86). Interestingly, it has been documented that $CD8^+$ T cell infiltration in MPTP-treated hemi-parkinsonian monkeys could be mitigated by inhibition of NFκB in microglia (86), emphasizing the importance of crosstalk between myeloid cells and lymphocytes in neuroimmune processes. Perhaps more relevant than reports of cytotoxic T cell involvement in MPTP models are studies of these cells in individuals with IBD, which includes both Crohn's disease (CD) and ulcerative colitis (UC). Abundant cytotoxic $CD8^+$ T cells are found in affected gut tissue of Crohn's and UC patients in active stages of disease (87), and enhanced activation of $CD8^+$ T cells in peripheral blood has been documented in CD patients (88). This phenotype appears to be even more prominent in UC, and levels of activated $CD8^+$ T cells in peripheral blood correlate with measures of UC-associated inflammation in blood and feces and are a reliable indicator of an active stage of disease (89, 90). DSS colitis models are considered to most closely resemble the pathology of UC, and they are known to induce accumulation of $CD8^+$ T cells in the colon, particularly in males (60). This study found significantly elevated *Cd8b* expression in colon tissue of male mice following acute colitis but more modest changes in females which only reached statistical significance in females lacking RGS10.

In summary, a novel finding of this study is that DSS colitis produces $CD8^+$ T cell infiltration in both the colon and the brain in males and that the degree of *Cd8b* expression in the brain is related to *Ifng* expression and to levels of TH in the striatum. The fact that these changes are observed at the mRNA level indicates that the T-cell associated immune response is still ongoing several weeks after induction of acute colitis while any gene expression changes related to myeloid cells are no longer detectable. This cytotoxic T cell response may represent a key mechanism by which colitis increases susceptibility to neurodegenerative disease. Further studies will be needed to determine whether $CD8^+$ T cells are in fact direct mediators of neuropathology and whether depletion of this immune cell subset affords neuroprotection against colitis-induced degeneration. Additionally, future studies that address the potential interactions of colitis, $CD8^+$ T cells, and human α-synuclein will be essential to further dissect the gene-environment interplay and the role of the gut-brain-immune axis in PD pathophysiology.

3.5 Figures and Tables

Score	Weight Loss (% baseline)	Feces Consistency	Fecal blood
0	Gain – 1.99%	firm, dry, well-formed pellets	no blood detected
1	2.0-7.99%	soft, moist, loose pellets	
2	8-13.99%	semi-liquid feces, no rectal adherence	Positive hemoccult test
3	14-19.99%		Visible blood in fecal smear
4	Over 20%	liquid feces, rectal adherence	Visible bleeding from rectum

Table 10. Criteria for calculation of disease activity index for colitis

Antibody	Manufacturer	Catalog Number	Concentration/ Dilution
Gt anti-RGS10	Santa Cruz	sc-6206	1:500; 1:200
Ms anti-β-actin	Santa Cruz	sc-47778	1:1000
Rb anti-CD68	Abcam	AB125212	1:200
Rb anti-PGP9.5	Millipore	AB1761-I	1:200
Rt anti-CD19	eBioscience	14-0193-85	1:500
Rt anti-CD3	Biolegend	100202	1:500
Rb anti-CD4	Abcam	AB183685	1:500; 1:250
Rt anti-CD8b	eBioscience	14-0083-82	1:500
Rb anti-NFκB p65	Santa Cruz	sc-372	1:1500
Rb anti-TH	Millipore	AB152	1:1500 western 1:1000 staining
Rt anti-DAT	Millipore	MAB369	1:5000
Rb anti-VMAT2	*Shared by WMC*	Lohr, *et al*. 2014 (63)	1:1000
Gt anti-Rb-**Biotin**	Vector Laboratories	BA-1000	1:500
Rb anti-TH phospho-Ser40	PhosphoSolutions	P1580-40	1:1000
Gt anti-Ms IgG-**HRP**	BioLegend	405306	1:2000
Gt anti-Rb IgG-**HRP**	Jackson ImmunoResearch	111-035-144	1:2000
Gt anti-Rt IgG-**HRP**	Jackson ImmunoResearch	112-035-006	1:2000
Dk anti-Gt IgG-**AF488**	ThermoFisher Scientific	A-11055	1:500, 1:1000
Dk anti-Rb IgG-**AF594**	ThermoFisher Scientific	A-21207	1:500, 1:1000
Dk anti-Rt IgG-**AF594**	ThermoFisher Scientific	A-21209	1:1000
Rt anti CD16/CD32	eBioscience	14-0161-85	1:100
Rt anti-Ly-6G-**PacBlue**	BioLegend	127611	1:100
Rt anti-CD11b-**PE-Cy7**	BioLegend	101215	1:200
Rt anti-CD4-**PE**	eBioscience	12-0041-81	1:100
Rt anti-CD8b-**APC-e780**	eBioscience	47-0083-82	1:100
Rt anti-CD45-**PerCP-Cy5.5**	eBioscience	45-0451-80	1:100
Ah anti-CD3-**PE-610**	eBioscience	61-0031	1:100
Rt anti-MHC-II-**APC**	Miltenyi Biotec	130-102-139	1:50
Rt anti-Ly-6C-**AF488**	eBioscience	53-5932-82	1:200
Rt anti-CD19-**BV650**	BioLegend	115541	1:100
Gt anti-Iba1	Abcam	AB5076	1:2000

Table 11. Antibodies used in this study

Conjugates are indicated in bold. Rb – rabbit, Ms – mouse, Gt – goat, Rt – rat, Ah – Armenian hamster, Dk – donkey, PGP – protein gene product, TH – tyrosine hydroxylase, DAT – dopamine transporter, VMAT2 – vesicular monoamine transporter 2, AF – Alexa Fluor

Gene	Forward Sequence (5'→3')	Reverse Sequence (5'→3')
RNA18SN5	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
PPIA	TGCCATCGCCAAGGAGTAG	TGCACAGACGGTCACTCAAA
Gapdh	CAAGGTCATCCATGACAACTTTG	GGCCATCCACAGTCTTCTGG
Ppia	TGGAGAGCACCAAGACAGACA	TGCCGGAGTCGACAATGAT
Chat	AAGGTCGGGTGGACAACATC	GTTTCTCAGAAGCCAGCACG
LCN2	TCACCCTCTACGGGAGAACC	CAGGGAGGCCCAGAGATTTG
Lcn2	TGGAAGAACCAAGGAGCTGT	GGTGGGGACAGAGAAGATGA
NOS2	CCTCCCCGAGGATCCCTCCC	CCTCCCCGCACTCCCTTGTG
Nos2	CAGGAGGAGAGAGATCCGATTTA	GCATTAGCATGGAAGCAAAGA
PTPRC	CATCACAGCGAACACCTCAGATGC	GCGCTTCCAGAAGGGCTCAGA
Ptprc	TCATGGTCACACGATGTGAAGA	AGCCCGAGTGCCTTCCT
CD8B	GACAGTGGCATCTACTTCTG	AAGGAAATCAACCACACTCA
Cd8b	GCTGTCCTTGATCATCACTCTCA	ACTAGCGGCCTGGGACATT
SNCA	CAGGAAGGAATTCTGGAAGAT	TAGTCTTGATACCCTTCCTCA
Snca	AAATGTTGGAGGAGCAGTGG	GAAGGCATTTCATAAGCCTCA
Th	TTGGCTGACCGCACATTT	GCCCCCAGAGATGCAAGT
Cd4	GTGAGCTGGAGAACAGGAAAGAG	GGCTGGTACCCGGACTGA
Ifng	CAAGTTTGAGGTCAACAACC	TCTTATTGGGACAATCTCTTCC
H2-Ab1	CAGGAGTCAGAAAGGACCTC	AGTCTGAGACAGTCAACTGAG
Tnf	CTGAGGTCAATCTGCCCAAGTAC	CTTCACAGAGCAATGACTCCAAAG
Il1b	ATCTTTGAAGAAGAGCCCAT	CCTGTAGTGCAGTTGTCTAA
Il6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA
Gfap	TGCTGGAGGGCGAAGAAA	CGGATCTGGAGGTTGGAGAA
Tlr4	ACTGTTCTTCTCCTGCCTGACA	TGATCCATGCATTGGTAGGTAATA

Table 12. qPCR primers used in this study

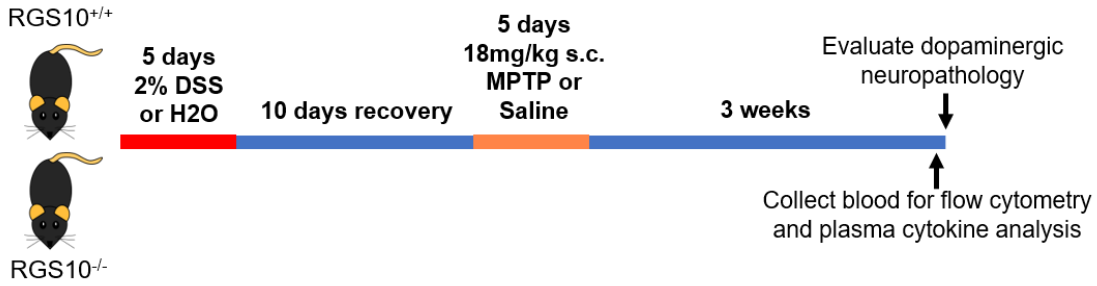

Figure 8. Mouse Experiment Design

RGS10 – regulator of G-protein Signaling 10, DSS – dextran sodium sulfate, MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

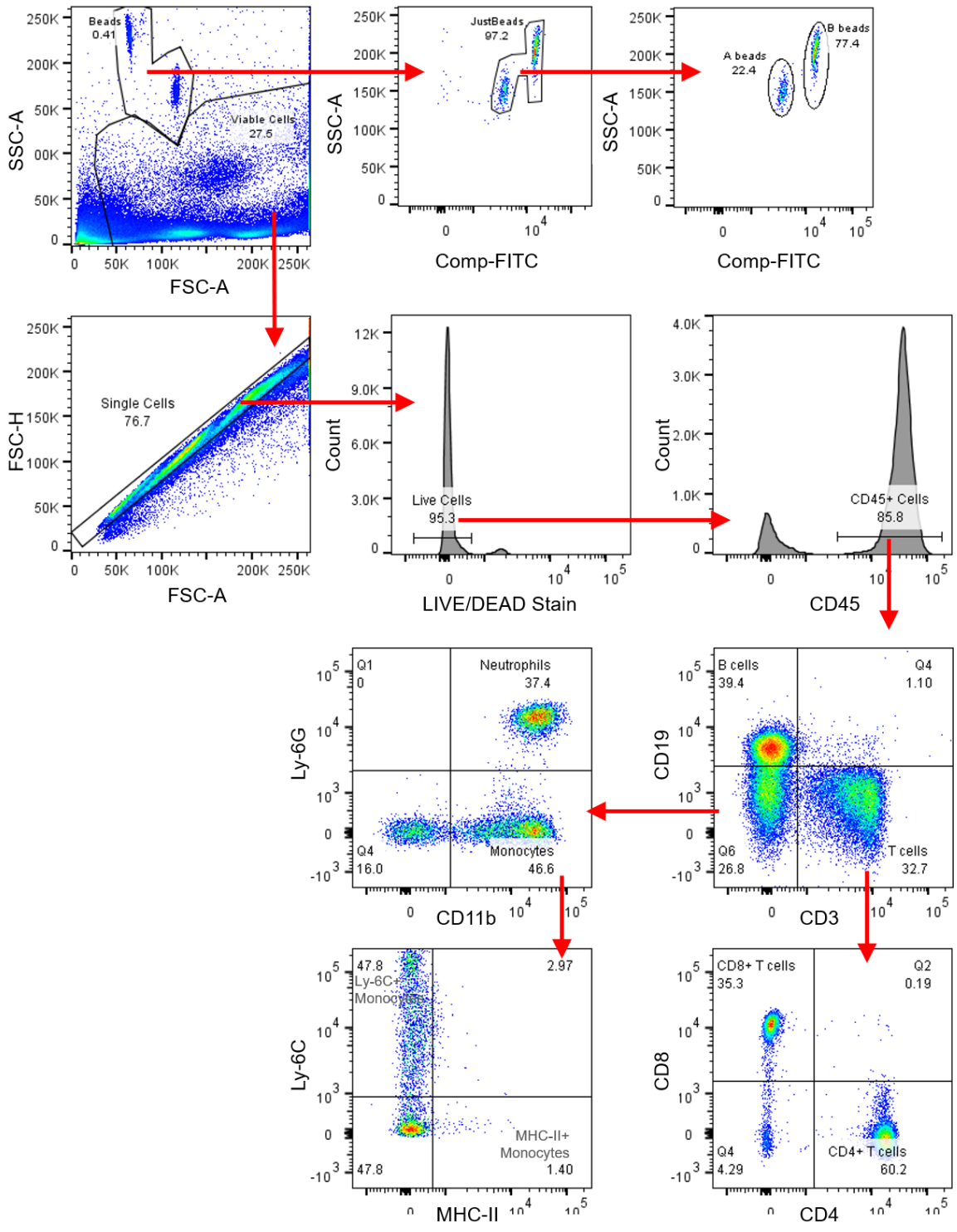

Figure 9. Gating strategy for mouse PBMCs

Performed in FlowJo 10.4.2 software.

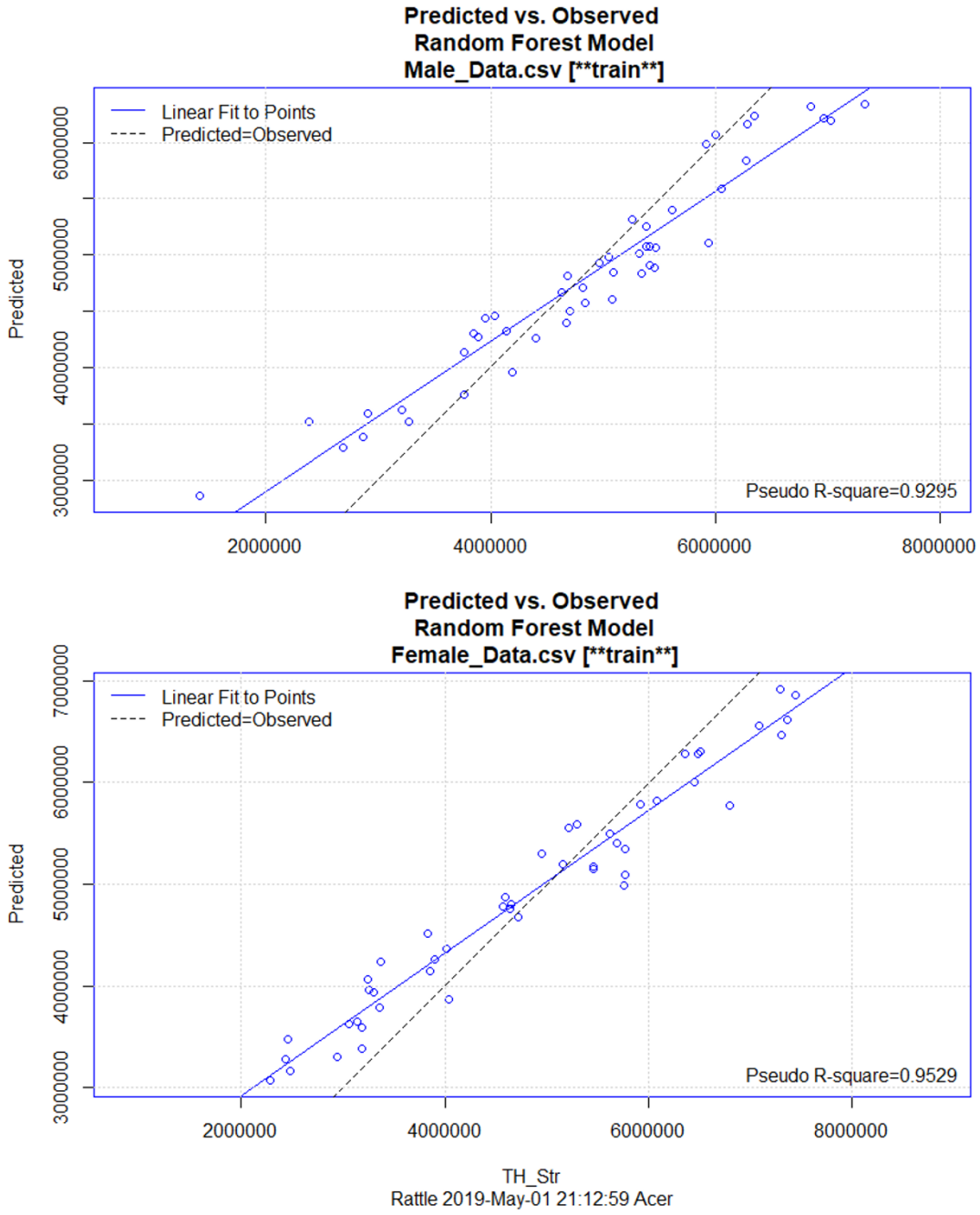

Figure 10. Random forest models are accurate representations of the datasets

Association between striatal TH values predicted by random forest models trained on these datasets and actual values in the datasets (target=TH protein in striatum, method=regression, n=55, number of trees=300, number of variables sampled for each tree=9).

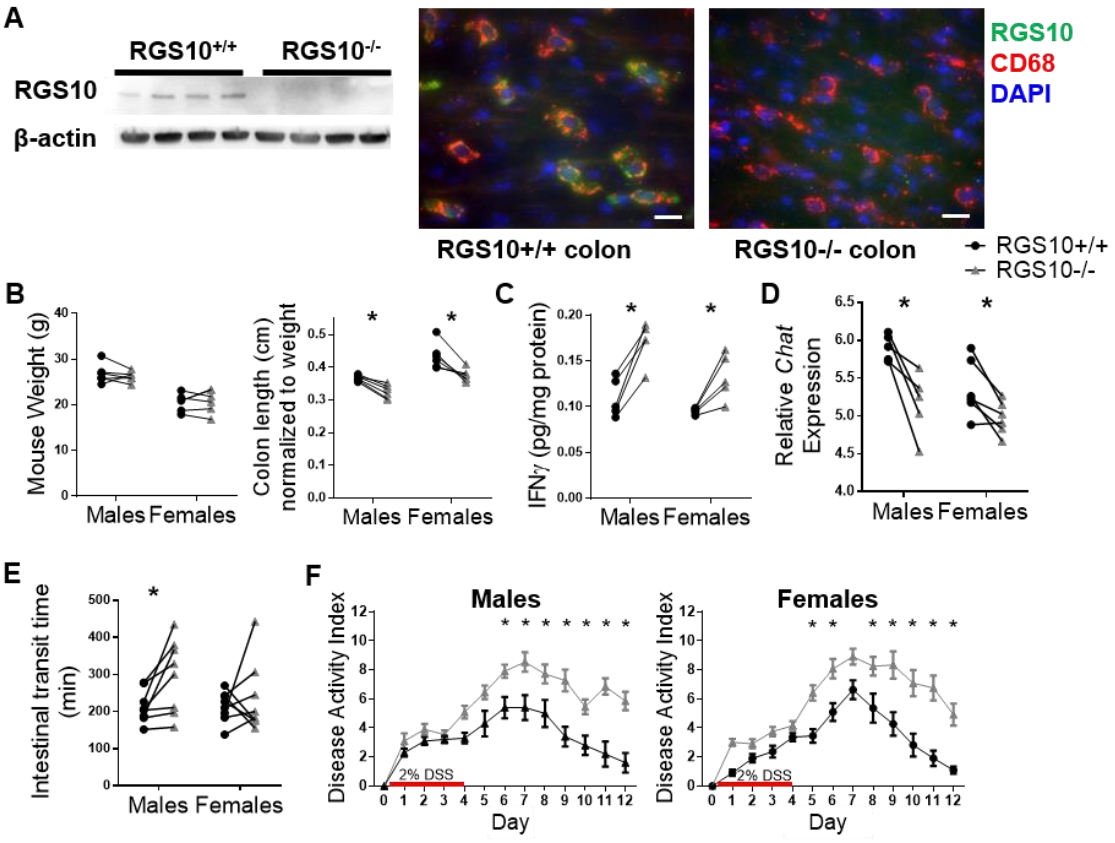

Figure 11. RGS10 deficiency induces intestinal inflammation and dysfunction and increases sensitivity to DSS colitis

A) RGS10 protein and RGS10^{+} cells (green) which are also CD68^{+} (red) in WT mouse colon tissue counterstained with DAPI, 40x, scale bar = 20μm. **B)** Mouse weight and colon length normalized to mouse weight (n=6), **C)** IFNγ concentrations determined by multiplexed immunoassay (n=5) and **D)** relative *Chat* mRNA levels (n=5-6) in distal colon tissue of 2-month-old male and female paired RGS10$^{+/+}$ and RGS10$^{-/-}$ littermates. **E)** Intestinal transit time determined by carmine red gavage in these mice (n=8). **F)** Disease activity indices of mice with DSS colitis. Two-way repeated measures ANOVA, genotype effect $p<0.05$ for all comparisons except mass; Sidak's *post hoc*. ***** indicates significant difference between genotypes.

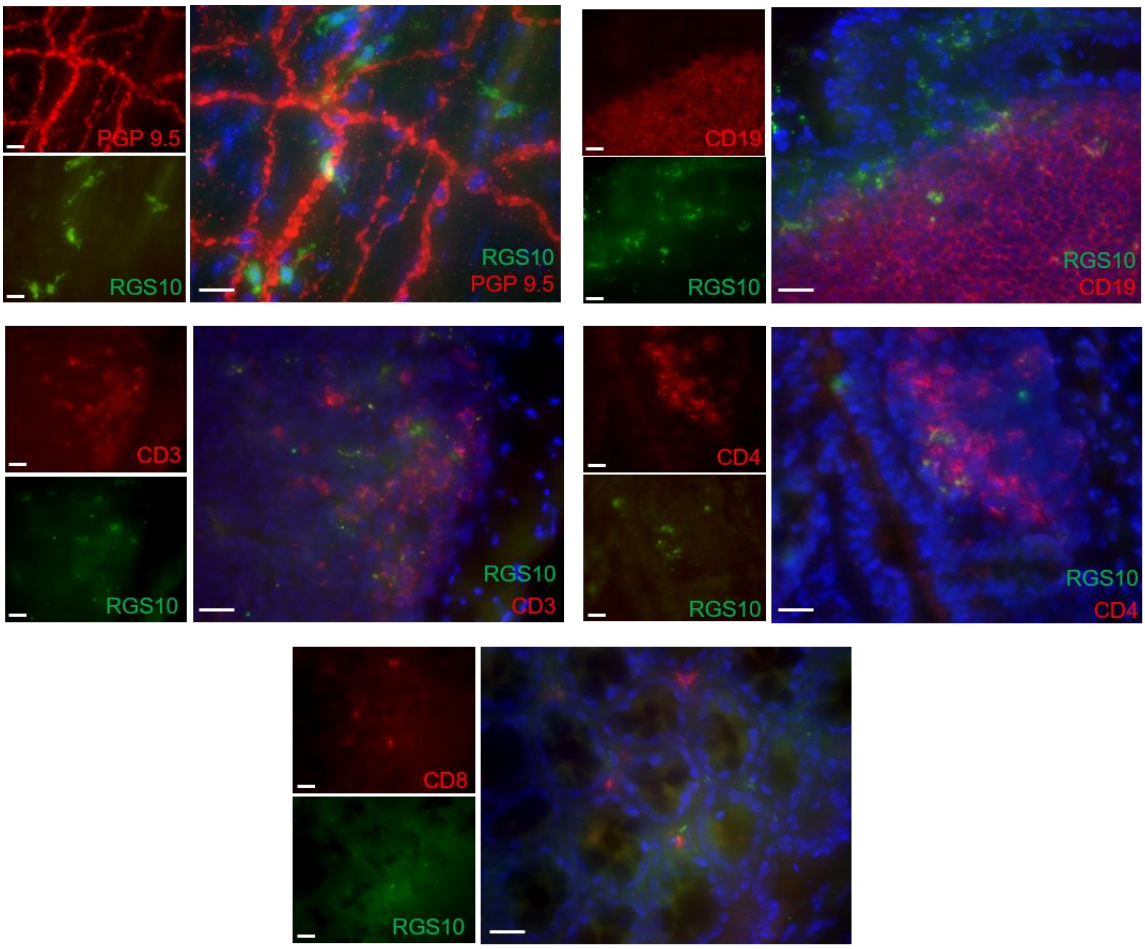

Figure 12. Neurons and lymphoid cells do not express RGS10 in the murine colon

Myenteric plexus was peeled from fixed colon tissue of RGS10-/- or RGS10+/+ mice and probed for RGS10 and PGP9.5. Frozen sections were probed for RGS10 and immune cell markers CD19, CD3, CD4, and CD8 (40x magnification, scale bar=20μm). Tissue was counterstained with DAPI.

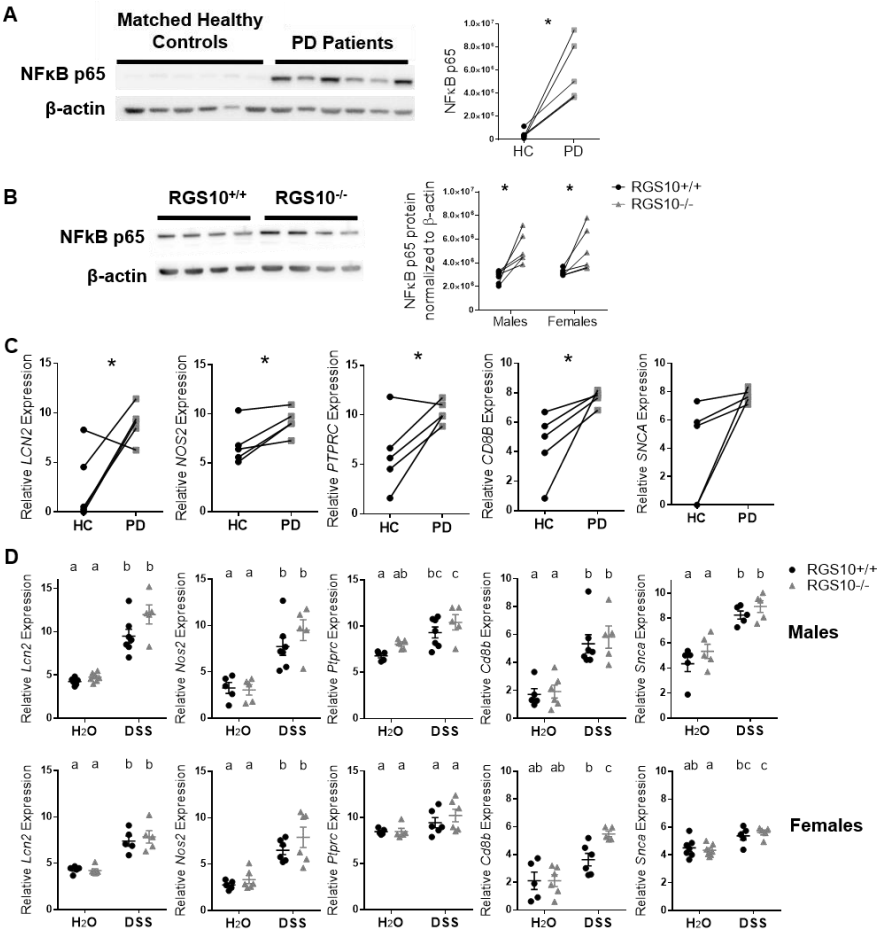

Figure 13. Colonic inflammation is present in PD patients and in mice with RGS10 deficiency and DSS colitis

A) NFκB p65 protein in sigmoid colon biopsies from PD patients and matched healthy controls (HC) (n=6, paired t-test, two-tailed, p=0.0052) and **B)** mouse distal colon tissue (n=6, two-way repeated measures ANOVA, genotype effect p<0.001, Sidak's *post hoc*). **C)** Relative mRNA levels in colon biopsies (n=5, paired t-test, two-tailed, p<0.05 for all except *SNCA* for which p=0.07). **D)** Relative mRNA levels in distal colon tissue from mice given H_2O or 2% DSS (5d) followed by water (5d) (n=5-7, two-way ANOVA, treatment effect p<0.05 for all, genotype effect p<0.05 for male *Lcn2* and *Ptprc*, Tukey's *post hoc*). Letters above groups and * indicate results of t-tests or *post hoc* tests with no shared letters or an * indicating significant differences between groups.

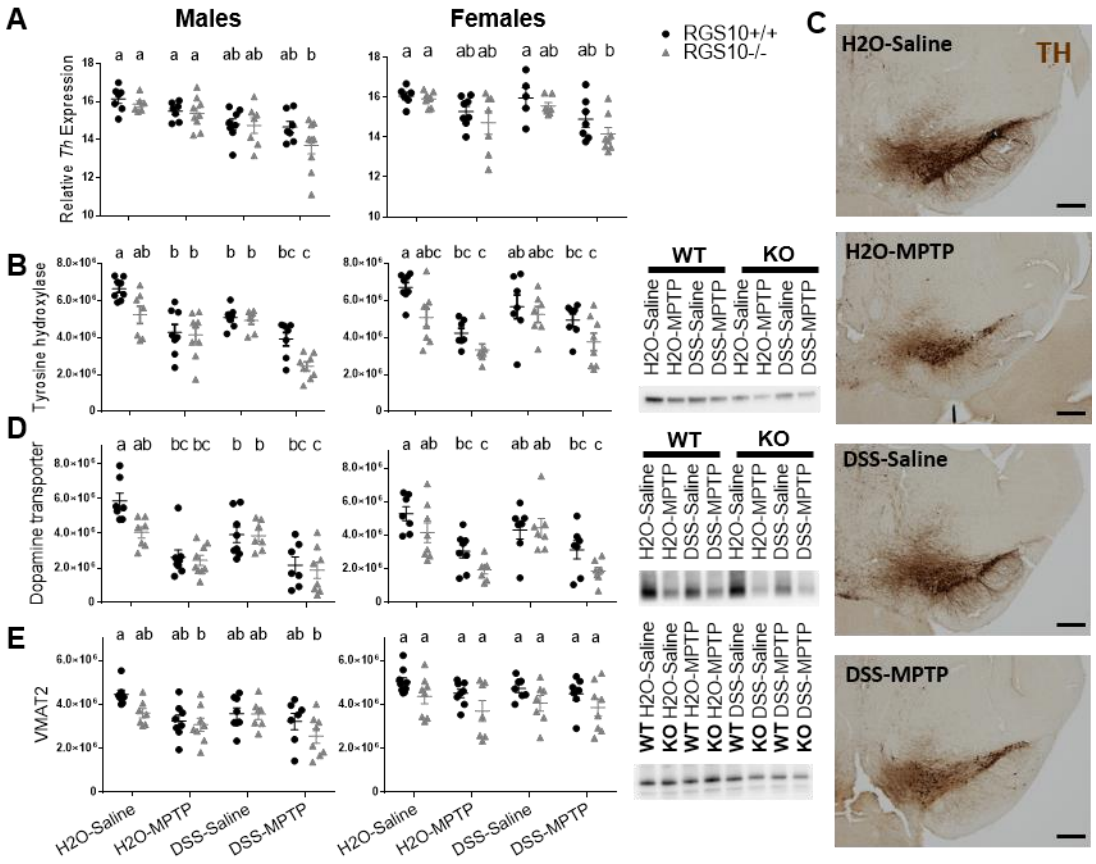

Figure 14. DSS colitis and RGS10 deficiency impact nigrostriatal dopamine pathways and increase susceptibility to MPTP

A) Relative mRNA levels encoding tyrosine hydroxylase (TH) in SNpc (n=5-8, two-way ANOVA, treatment effect $p<0.001$, Tukey's *post hoc*), B) representative images of TH^+ (brown) cells in the $RGS10^{+/+}$ SNpc (4x magnification, scale bar = 250μm), and C) TH, D) dopamine transporter, and E) VMAT2 protein in striatum normalized to total protein (n=7-9; two-way ANOVA, treatment effect $p<0.01$ for all except VMAT2 for females, genotype effect $p<0.05$ for all, Tukey's *post hoc*) from mice given H_2O or DSS followed by saline or MPTP. Letters above groups indicate *post hoc* results; no shared letters indicate significant differences between groups.

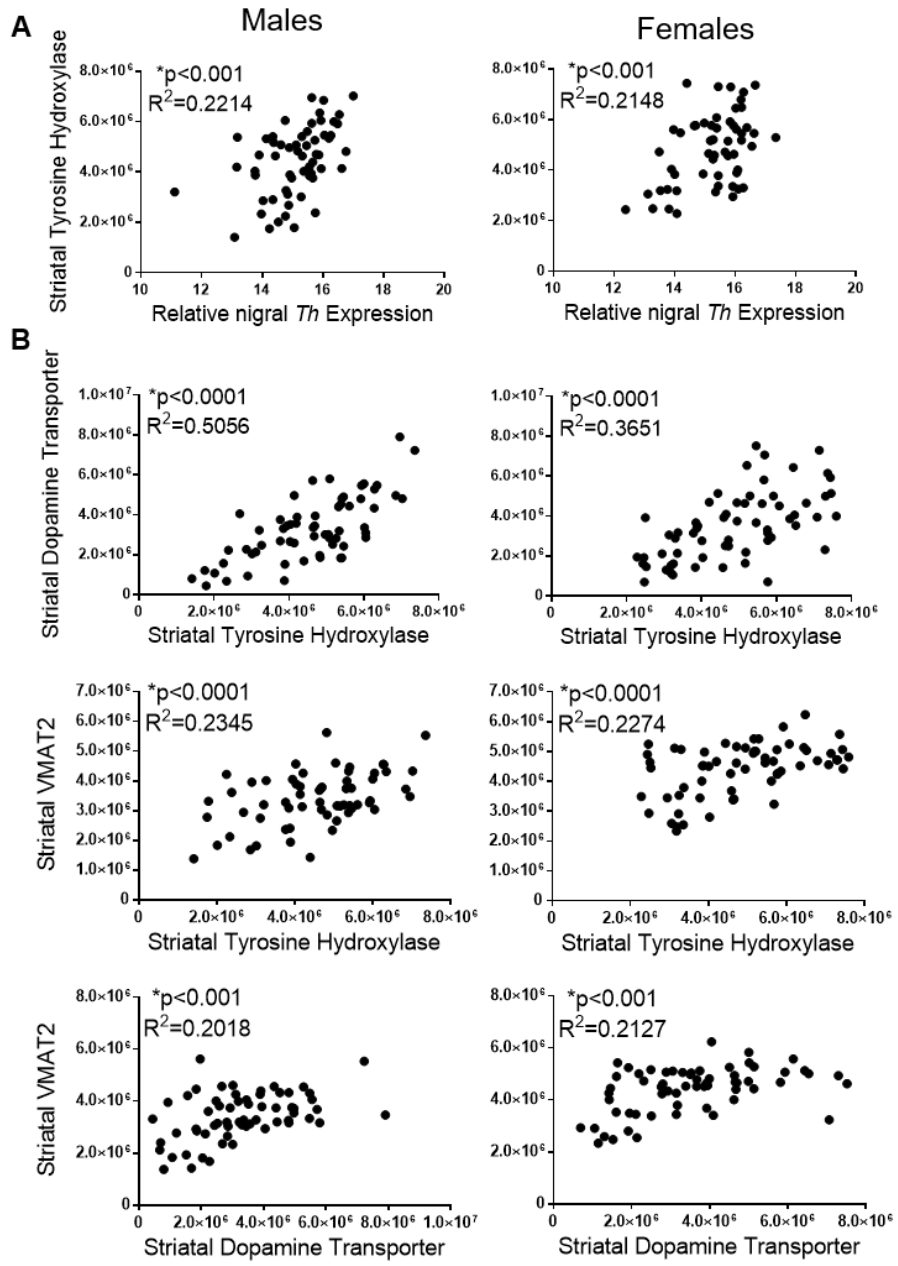

Figure 15. Levels of factors regulating dopamine production, packaging, and reuptake are significantly correlated

A) Relationships between relative mRNA levels encoding tyrosine hydroxylase (TH) in substantia nigra and TH protein in striatum and among **B)** TH, dopamine transporter, and VMAT2 in striatum from male and female mice in all experimental groups (n=58-63; Pearson's correlation)

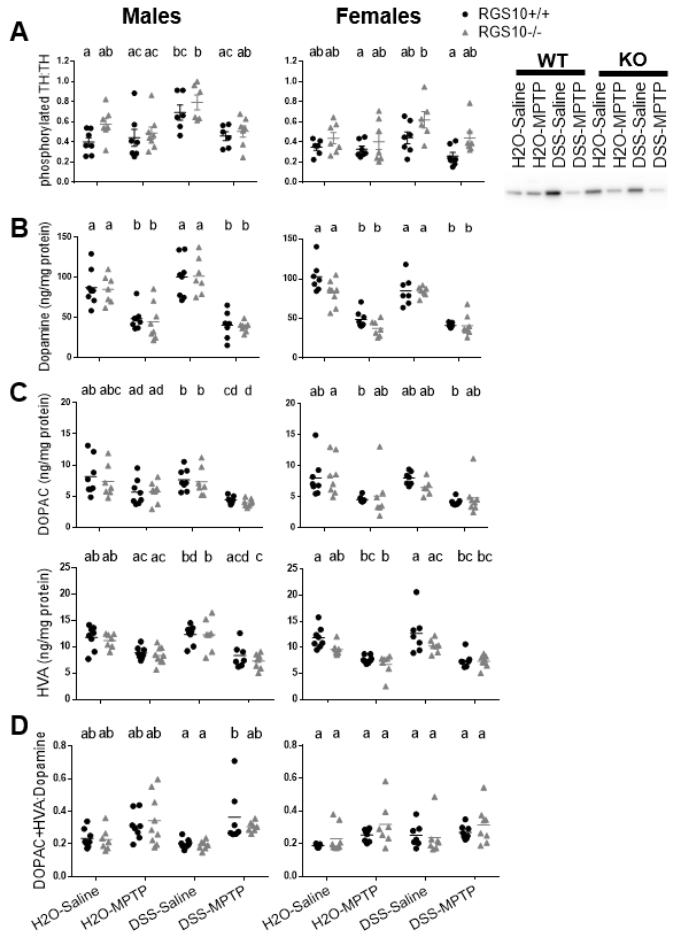

Figure 16. Increased tyrosine hydroxylase activity prevents dopamine deficiency after colitis

A) Ratio of tyrosine hydroxylase (TH) protein phorphorylated at serine 40, which increases the enzyme's activity, to total TH determined by western blot (n=7-9, two-way ANOVA, treatment effect and genotype effect $p<0.05$, Tukey's post hoc). B) Levels of dopamine, C) 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and D) the ratio of the sum of DOPAC and HVA to dopamine measured by HPLC in striatum from mice given H2O or 2% DSS followed by saline or MPTP (n=6-9, two-way ANOVA, treatment effect $p<0.05$ for all, genotype effect $p<0.05$ for dopamine and HVA in females, Tukey's post hoc). Letters above groups indicate post hoc results with no shared letters indicating significant differences between groups.

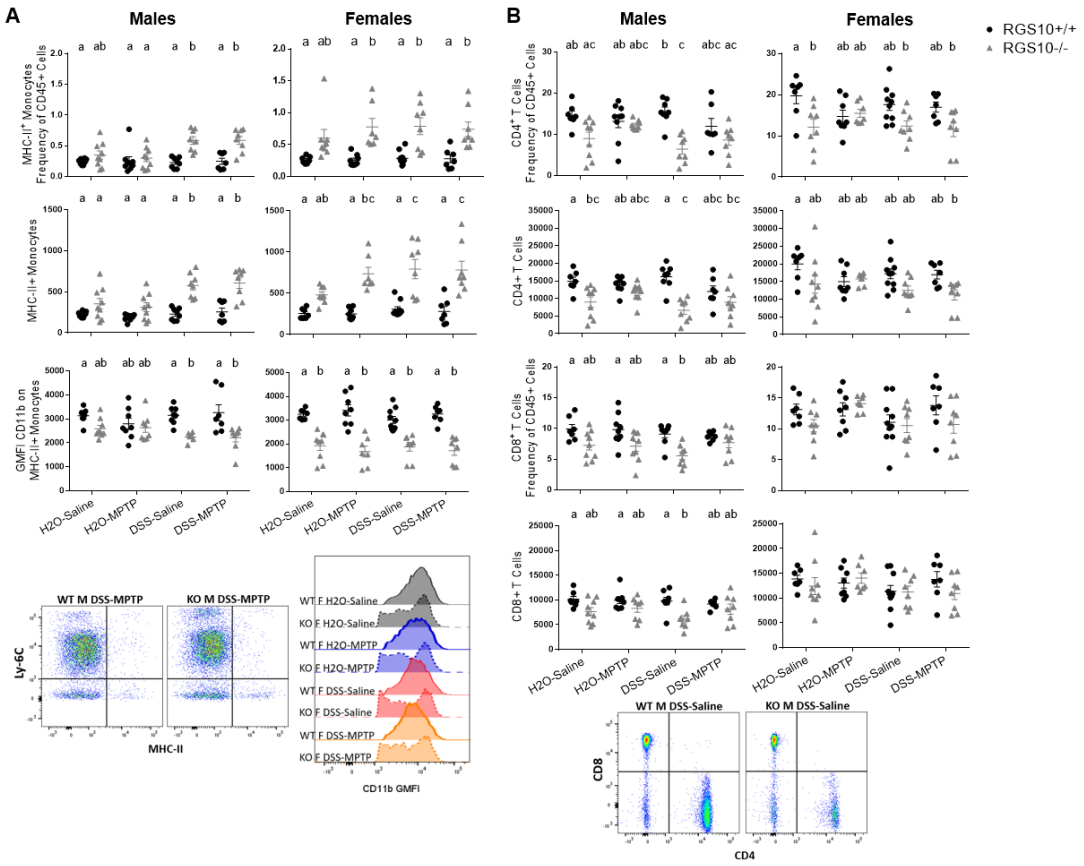

Figure 17. RGS10 deficiency impacts circulating non-classical monocytes and T cells, and colitis augments these effects

A) Frequency of CD45+ cells, counts, and geometric mean fluorescence intensity (GMFI) of CD11b for Ly-6C- MHC-II+ (non-classical) monocytes and B) frequency of CD45+ cells and counts of CD4+ and CD8+ T cells in peripheral blood mononuclear cells from mice given H2O or 2% DSS followed by saline or MPTP determined at endpoint by flow cytometry (n=7-10, two-way ANOVA, genotype effect $p<0.05$ for all except female CD8+ T cells, treatment effect $p<0.05$ for male MHC-II+ monocyte frequency and male and female MHC-II+ monocyte counts, interaction $p<0.05$ for male MHC-II+ monocytes and male CD4+ T cells frequency and counts, Tukey's post hoc). Letters above groups indicate post hoc results with no shared letters indicating significant differences between groups.

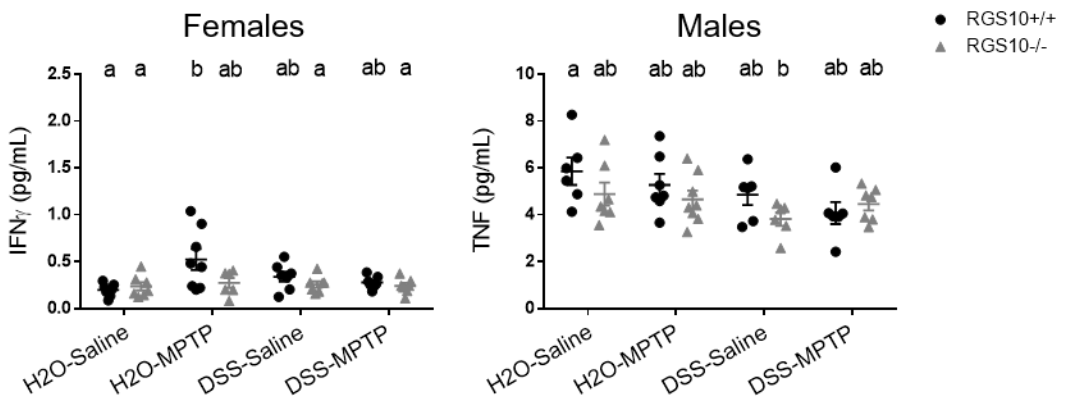

Figure 18. Minimal impact of genotype or treatment on plasma cytokines at experiment endpoint

Cytokines in plasma measured at endpoint by multiplexed immunoassay (n=6-8, two-way ANOVA, treatment effect $p<0.05$, Tukey's post hoc). Letters above groups indicate post hoc results with no shared letters indicating significant differences between groups.

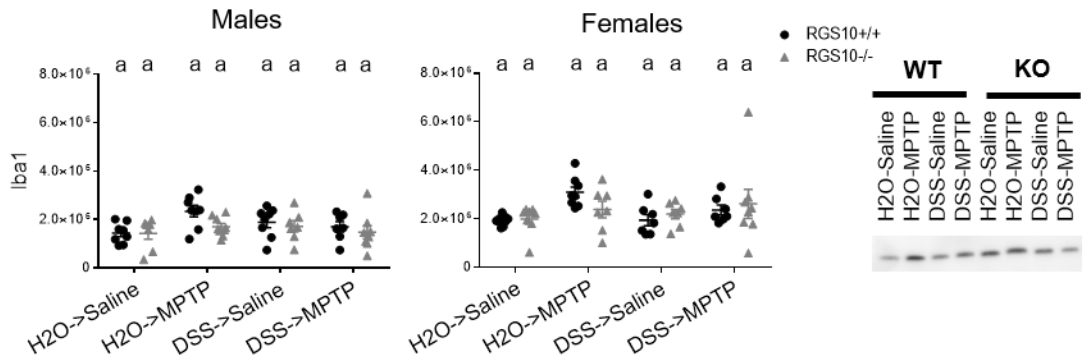

Figure 19. No differences in Iba1 protein levels among experimental groups

Iba1 protein in striatum normalized to total protein (n=7-9; two-way ANOVA, treatment effect $p<0.05$, Tukey's *post hoc*) in striatum and substantia nigra tissue sections from mice given H_2O or 2% DSS followed by saline or MPTP. Letters above groups indicate *post hoc* results with no shared letters indicating significant differences between groups.

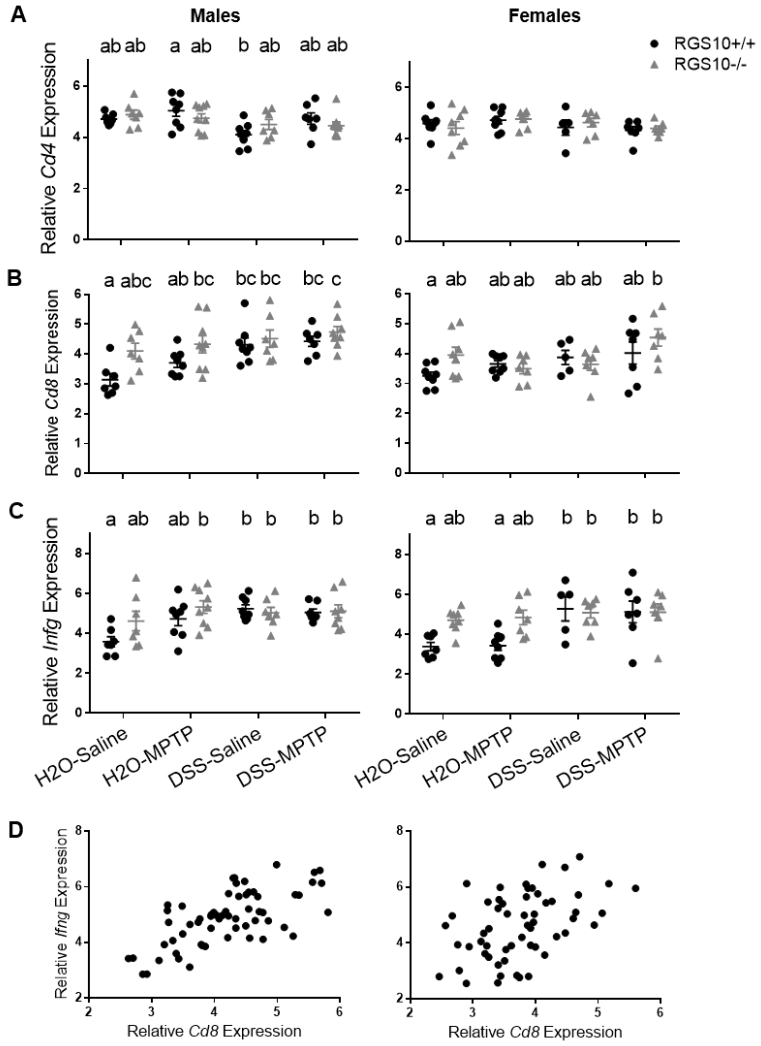

Figure 20. Colitis and RGS10 deficiency result in CD8$^+$ T cell infiltration and elevated *Ifng* expression in the substantia nigra

A-C) mRNA levels determined by qPCR in the substantia nigra of mice given H_2O or 2% DSS followed by saline or MPTP (n=5-9, two-way ANOVA, treatment effect $p<0.05$ for all except *Cd4* for females, genotype effect $p<0.05$ for *Cd8* for males and *Ifng* for females, Tukey's *post hoc*). Letters above groups indicate *post hoc* results with no shared letters indicating significant differences between groups. **D)** Significant correlation between nigral *Cd8* and *Ifng* (n=56-62, Pearson's correlation, $p<0.001$, $R2=0.4445$ for males and 0.2057 for females).

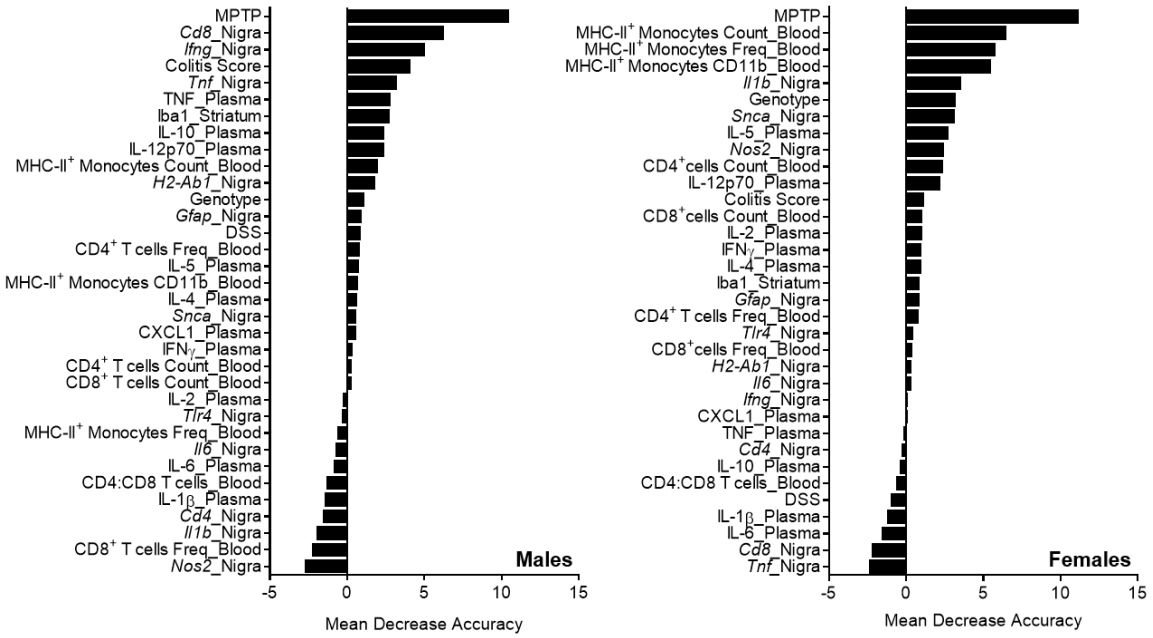

Figure 21. Factors most highly associated with striatal TH levels differ by sex

Ranking of variable importance according to the mean decrease in accuracy of the random forest model for males and females when the variable is excluded (target=TH protein in striatum, method=regression, n=55, number of trees=300, number of variables sampled for each tree=9).

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CHAPTER 4: DISCUSSION, CONCLUSIONS, AND FUTURE DIRECTIONS

Over the years, our understanding of Parkinson's disease (PD) has evolved from viewing the disease as a loss of midbrain neurons giving rise to motor deficits to recognition of a multi-system disorder with central and peripheral, motor, non-motor, and pre-motor manifestations, many of which involve the gastrointestinal (GI) tract. A preponderance of evidence now suggests that the GI tract is not only affected in PD, but that this may be the site where pathology initiates decades before progressing from the enteric to the central nervous system. In late 2011 and 2012, the first studies in which authors speculated on potential connections between oxidative stress or inflammation and intestinal manifestations of PD were published (1, 2), and in 2015, the concept that intestinal inflammation could contribute mechanistically to parkinsonian neurodegeneration began to be clearly articulated in the literature (3, 4). There was little evidence then, however, and even at the time of publication of our proposed model for inflammation-driven, gut-originating PD (5) at the beginning of 2017, that abnormal GI immune activity occurred in PD.

Since then, several studies have reported significant increases in proinflammatory cytokines (6) and other indicators of inflammation (7) as well as abnormal immune cell infiltration (8) in colon tissue or feces from PD patients compared to age- and sex-matched healthy controls. One of these was our work describing increases in four proinflammatory cytokines and chemokines in stool from PD patients that were detectable after accounting for the influence of numerous potential confounders and covariates (9). We also found increased expression of genes related to inflammation, oxidative stress, and immune cells in colon biopsies from PD patients compared to matched healthy controls, and we showed that expression of these same factors was increased by experimentally-induced colitis in mice. Concordantly, epidemiological studies have recently been published reporting that irritable bowel syndrome

(IBS) or inflammatory bowel disease (IBD) patients are at a higher risk for PD (10-14). Incidentally, we also observed a higher incidence of intestinal disease including IBS, IBD, and broadly categorized colitis in our cohort of PD patients compared to controls (9). We also demonstrated that the induction of experimental colitis in a mouse model was capable of mediating neurological and neuroinflammatory effects in the midbrain that persisted weeks after the resolution of GI symptoms. As a result of this growing body of evidence, the field now generally acknowledges that gut inflammation, and not just gut dysfunction, is a common symptom associated with PD that likely contributes to neuroinflammation and neurodegeneration. Further studies aimed at directly testing this idea are certainly warranted.

These studies describing measures of GI inflammation in PD, particularly those that evaluate levels of particular proinflammatory mediators, also begin to address the next crucial challenge in defining the role of the gut-brain axis in PD pathogenesis, which is to identify particular cell types, immune pathways, and molecular factors that are involved rather than continuing to rely on the broad descriptor "inflammation." Many of our findings and others in the literature converge on one pathway – signaling through toll-like receptor 4 (TLR4) activates the transcription factor nuclear factor kappa B (NFκB) and induces expression of genes encoding many proinflammatory factors. Lipopolysaccharide (LPS) or endotoxin is a bacterial product that ligates TLR4, and its administration peripherally or directly into the brain has been used for years to induce parkinsonian pathology in rodent models through mechanisms involving activation of microglia (reviewed by (15)). Increases in levels of LPS in circulation resulting from increased gut permeability have been proposed as a contributing factor to neuroinflammation in PD, and measurements of the more stable LPS-binding protein (LBP) as a surrogate for LPS in human plasma have indicated enhanced LPS-associated signaling in PD

patients (1, 8, 16). Perez-Pardo *et al*. recently reported greater accumulation of $TLR4^{+}$ cells in the colonic mucosa of PD patients relative to controls and suggested that dysregulation of this pathway could contribute to pathological crosstalk between the gut and brain in the development of PD (8). They also measured lower mRNA expression associated with toll interacting protein (TOLLIP), an inhibitor of TLR4 signaling (17), and higher mRNA expression associated with interleukin-1 receptor-associated kinase 2 (IRAK2), a downstream signaling molecule necessary for LPS/TLR4 activation of NFκB through the adaptor protein Myeloid Differentiation Primary Response 88 (MYD88) (18), in the PD colon (8). We discovered marked increases in levels of NFκB in colon biopsies from PD patients, and we showed in mice that loss of the protein Regulator of G-protein Signaling 10 (RGS10), which is downregulated in response to LPS (19, 20), increases NFκB levels, induces low-grade intestinal inflammation as well as more severe and persistent colitis after exposure to dextran sodium sulfate (DSS), and increases neuropathology of the nigrostriatal pathway following DSS colitis. We also observed higher levels of IFNγ in colon tissue in $RGS10^{-/-}$ mice, and treatment with this cytokine is known to upregulate expression and activity of numerous components of the TLR4-NFκB signaling pathway in myeloid cells, priming these cells to respond more vigorously to stimulation with LPS (21). We and others have also reported increases in the expression of cytokines in the colon in PD patients that are regulated by the transcription factor activity of NFκB downstream of TLR4 signaling (6, 8, 9).

To our knowledge, no associations with PD risk have been found relating to genetic polymorphisms or mutations in TLR4, IRAK2, TOLLIP, NFκB, or RGS10. It may be, therefore, that the altered levels of various components of this pathway reflect the accumulation of immune cells actively engaged in inflammatory responses in the gut rather than inherent variations in

expression or activity of signaling molecules that predispose to PD-related GI or neurological pathology. Nonetheless, these studies do strongly suggest that this pathway is highly active in PD, and it may represent a tractable therapeutic target. Numerous studies have demonstrated the neuroprotective effects derived from inhibition of this pathway in animal models of parkinsonian neurodegeneration (22-24). Of course, elements of this pathway such as NFκB interface with numerous signaling cascades and are fundamental elements of immune responses, so further research is needed to inform the design of targeted therapies that could ameliorate PD pathology without causing severe immunosuppression. It will be important to determine which cell types are involved, to confirm that the entire pathway is more active in PD rather than that a particular component is atypically expressed or active, and to evaluate whether PD-related effects on this pathway are cell-intrinsic or a consequence of higher concentrations of TLR4 ligands in the gut environment, perhaps as a result of dysbiosis.

The repeated identification of the TLR4 signaling pathway – which is primarily active in myeloid cells (25) – in gut and brain pathology in PD and PD-related animal models emphasizes the importance of these cells in the disease process and indicates that they may be key mediators of gut-brain crosstalk in PD. The activities of peripheral monocytes may be particularly relevant. The alleles known to be related to PD risk are more abundant in monocytes than other circulating immune cell types (26), and recent studies have identified gene expression profiles in monocytes from PD patients, including patients in the early stages of the disease, that were distinct from controls (27, 28). One study identified LPS as the upstream factor most highly associated with the PD-related signature and demonstrated that monocytes from PD patients exhibited hyperactivity in response to LPS (27). The other study identified genes encoding NFκB, MyD88, and TNF – components of the LPS-TLR4-NFκB pathway – and also MHC-II, a key molecule in

the presentation of antigen to and activation of T helper cells (28). Both IFNγ (29) and LPS (30) stimulate increased expression of MHC-II in myeloid cells, and we observed more MHC-II$^+$ monocytes in the peripheral blood of mice lacking RGS10, particularly after DSS treatment. Our supervised machine learning model also showed that the abundance of these cells in females was the best predictor (other than MPTP treatment) of levels of striatal tyrosine hydroxylase (TH), the enzyme which catalyzes the rate-limiting step in the biosynthesis of dopamine. Infiltration of monocytes expressing MHC-II into the CNS has been observed in various models of neuropathology and neurodegeneration (31, 32), and these cells have been reported to be essential mediators of the transmission of inflammatory responses from the periphery to the brain and of the neurodegeneration that resulted (33, 34).

A key mechanism related to this may be production of the chemokine CCL2. CCL2 production by myeloid cells is upregulated in response to LPS-induced activation of NFκB (35), and it can also be produced by other cells including astrocytes and neurons under inflammatory conditions (36). Unusually high levels of CCL2 are found in the blood of PD patients relative to healthy controls (27). Both myeloid cells and T cells express receptors for CCL2, and increases in CCL2 production in the CNS result in the infiltration of monocytes as well as regulatory and interferon gamma (IFNγ)-producing T cells into the brain (36-38). While studies suggest that production of CCL2 in the CNS is not sufficient to produce overt neuropathology (37), in models of neurodegenerative disease, blockade of CCL2 signaling pathways significantly mitigates CNS immune cell infiltration, neuroinflammation, and neuron loss (36, 38-40).

Intriguingly, our research revealed that the infiltration of CD8$^+$ cytotoxic T cells into the midbrain after colitis and the associated production of IFNγ were related to levels of TH in the striatum of male mice, highlighting this as a potential mechanism that could link GI immune

responses with PD-related neuroinflammation and neuropathology. While $CD8^+$ T cells have been observed in the brains of PD patients (41) along with abnormalities in the frequency and activation of circulating $CD8^+$ T cells (42-44), studies have focused primarily on the potential contributions of monocytes and $CD4^+$ T helper cells to gut-brain crosstalk in PD (8, 45). It is known, however, that cytotoxic T cells are involved in IBD. Activated $CD8^+$ T cells accumulate in affected intestinal tissue (46), and these cells are also detected in the blood and can predict whether an individual is in an active stage of disease (47). Corresponding findings have been reported in DSS colitis, and more $CD8^+$ T cells are found in colon tissue from male mice compared to females (48). These findings were reflected in our DSS colitis experiment as well, with increased colonic *Cd8b* in DSS-treated males, but significant increases with DSS only in females that also lacked RGS10. We also observed significantly higher levels of *CD8B* in colon tissue from PD patients compared to matched controls.

Together, these findings suggest that $CD8^+$ T cells could be mediators of colitis-associated effects that increase the risk for development of PD in humans. Furthermore, the anti-TNF therapies that were reported to substantially reduce the risk of PD in IBD patients have also been shown to reduce the frequency and cytotoxic activity of $CD8^+$ T cells (49, 50). Future studies will be needed to confirm our findings and to expand them with evaluation of cytotoxic T cell activation and activity in colitis models. Colitis experiments and examination of dopaminergic neuron health and function in the midbrain in the context of $CD8^+$ T cell depletion could also provide insight on the role these cells might play in the development of neuropathology. Finally, evaluation of *post mortem* brain tissue from individuals with IBD to determine whether $CD8^+$ T cells are present in higher numbers or in a more activated state than

in age-matched controls without GI or neurological disease would help to confirm the applicability of these experimental colitis experiments to human physiology.

Despite the contribution of our work and that of others in identifying potential immunological mechanisms mediating gut-brain connections in PD, it cannot be argued that a specific gut inflammatory profile of PD has been established. While several studies have found increased levels of proinflammatory cytokines in colon tissue or stool from PD patients, different cytokines have been identified in each study (6, 8, 9). Fecal calprotectin, found to be elevated in PD patients compared to controls (7), is a common indicator of intestinal inflammatory processes, as is mRNA expression of LCN2, identified in our study. This suggests that it will be difficult to pinpoint particular biomarkers that constitute a peripheral inflammatory signature that is specific to PD, as has been proposed. This endeavor is further complicated by the powerful effect of sex and other demographic and lifestyle factors on GI immune responses and on dopaminergic neuropathology which is consistently demonstrated by the studies described in this dissertation (9). Our work illustrates that failing to account for these variables in human studies is likely to obscure PD-associated differences, providing a potential explanation for some of the seemingly contradictory findings in the literature regarding GI pathology in PD. Our mouse experiments show that differences in sex and genotype, even with respect to a single gene, result in highly disparate responses to the same inflammatory challenge. This renders it still more difficult to determine a particular threshold at which levels of an inflammatory factor could be considered indicative of PD or its imminent manifestation. Yet, these findings do provide insight into the pathological mechanisms occurring in the disease, and they could be used in conjunction with other known genetic or environmental risk factors to identify individuals who might be at a higher risk for the development of PD.

For this to be possible, however, it will be essential to improve our understanding of the dynamics of intestinal inflammatory phenotypes in PD. While prospective and retrospective studies indicate that GI pathology in subjects with PD manifests before motor impairment, it is still unclear whether GI inflammation develops before and contributes to neuron degeneration and loss or whether it arises in response to damaged and dying neurons. Evidence such as higher incidence of PD developing among individuals who had already been diagnosed with IBD, our finding that the levels of inflammatory factors in stool in PD patients did not increase with increasing duration of disease, and studies like ours which demonstrates that the induction of damage and inflammation specifically in the intestine can impair neuron health in the brain support the former hypothesis. Longitudinal and prospective studies evaluating indicators of inflammation and neuropathology in the gut and the brain would provide the information necessary to more conclusively address this question.

It has also not been well established that the GI dysfunction which is observed in clinical PD as well as its prodromal stages results from intestinal inflammation, though the symptoms are consistent with those that could be expected under conditions of low-grade gut inflammation. In our study of fecal inflammatory factors in human subjects, we did not identify any significant direct associations between levels of an inflammatory mediator in stool and subject reports of GI dysfunction. Other studies have also reported finding no such associations (7). A key confounder in these efforts may be the unreliability of self-reports of gut dysfunction, as studies have demonstrated marked differences in the incidence of conditions such as constipation in PD patients when determined by self-reporting versus objective measurements (51). Detailed studies in human subjects which utilize objective measures of GI function and standardized methods for

evaluating PD-related GI pathology, such as the detection of potentially pathological forms of α-synuclein, will be needed to thoroughly evaluate associations with mediators of inflammation.

Answering these questions regarding the mechanisms, timing, and functional effects of GI inflammation and its relationship with onset of clinical PD symptoms is critical because the involvement of intestinal immune responses in PD presents opportunities for the development and application of novel diagnostics and earlier therapeutic interventions. At present, PD is typically diagnosed only after extensive neurodegeneration produces the characteristic motor impairments, and treatment at this stage primarily seeks to ameliorate those symptoms by supplementing dopamine levels. Similarly, efforts to therapeutically target inflammation, enteric or otherwise, at advanced stages of PD might mitigate some symptoms of autonomic dysfunction and could perhaps even affect progression of the disease slightly, but it is unlikely to halt and will not reverse progressive neurodegeneration. A more promising strategy is to treat inflammation in the earliest stages of PD before neurological damage is sufficiently advanced to produce motor symptoms. If, as current research suggests, chronic intestinal inflammation can initiate processes which ultimately result in neurodegeneration in the central nervous system (CNS), then promoting its rapid resolution or at least its suppression could delay or prevent loss of midbrain neurons and the onset of debilitating motor symptoms. Another advantage of this therapeutic approach is that immunomodulatory drugs could be effective at this early stage by acting on peripheral sites of pathology without the need to cross the blood-brain-barrier, eliminating a pharmacological challenge. Successful implementation of these measures would still necessitate identification of individuals experiencing forms of GI inflammation that increase their risk for development of PD, but studies have already identified one such population, namely individuals diagnosed with IBD. Our research indicates that even one acute experience of severe

colitis in conjunction with other risk factors can promote neuropathology and sensitize to the effects of a subsequent neurological insult, and other studies demonstrate the efficacy of immunosuppressant therapy in reducing this risk (12, 52). This emphasizes the importance of effectively managing this condition, not just to improve the present quality of life for the patient, but to reduce the risk of long-term neurological sequelae.

PD is a complex disorder that develops over decades as a result of the interaction of sex, genetics, and environmental and lifestyle factors. Its presentation varies widely among patients, and it is unlikely that any one proposed etiological mechanism will apply to all cases, but a growing body of research provides strong support for a gut-originating, inflammation-driven pathway of PD pathogenesis that is evident in at least a substantial subset of patients with sporadic PD. Research that enhances scientific understanding of these processes can contribute to the development of novel treatment and, most importantly, prevention strategies to reduce the societal burden of this disease.

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