The Impact of Bacteriophage on the Aging Brain and Inflammatory Response: Relevance to Parkinson's Disease

by

Sheryl Beauchamp

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Abstract

Parkinson's disease (PD) is associated with age and inflammation. New studies have found a link between gut dysbiosis and the prevalence of PD. Phage 936, which is found in dairy products, has been associated with disruptions in the gut microbiome and leaky gut causalities. The present thesis sought to assess the impact of phage 936 in young or old mice and whether the virus augments or diminishes the impact of an inflammatory (LPS) stimulus. To this end, an initial study was conducted to first determine if bacteriophage alone could actually produce some degree of measurable changes (e.g. neutrophil mobilization or change in cytokine or other circulating immune factors) within the brain. The main study of this thesis then involved young (4-5 months) vs old (15-16 months) mice receiving the phage 936 (or vehicle), followed by LPS (or vehicle) treatment. We then assessed peripheral gut and brain inflammatory changes, as well as assessed motor functioning and sickness. We hypothesized that the old mice that received both the LPS and phage 936 would display the greatest degree of inflammatory and neuronal pathology. However, it is possible that the bacteriophage would diminish the impact of LPS given that phages can neutralize endogenous bacteria and hence, might limit the inflammatory profile. Our findings provide evidence that phage alone does cause measurable changes in inflammatory biomarkers, both peripherally and centrally. We also determined that phage 936 caused behavioral changes, as evidenced by sickness scores and weight loss. It could therefore be concluded that our hypothesis was reasonable; both LPS and age did exacerbate the immunological changes produced by phage, thus producing detectable pathology in mice. We present for the first time, that phage 936, in aged mice may have complex effects that vary with the presence of inflammation (e.g. induced by LPS).

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List of Abbreviations

BBB= blood brain barrier

CNS= central nervous system

CX3CR1= Chemokine (C-X3-C) Receptor

GFAP= glial fibrillary acidic protein

IBA1 = ionized calcium-binding adapter molecule

IP= intraperitoneally

LPS = lipopolysaccharide

MFG-E8= Milk fat globule-EGF factor 8 protein

NADPH = nicotinamide adenine dinucleotide phosphate

NFkB = nuclear factor kappa-light-chain-enhancer of activated B cells

NGS = normal goat serum

PAMP = pathogen associated molecular pattern

PB = phosphate buffer

PBS = phosphate buffered saline

PD = Parkinson's disease

PFA = paraformaldehyde

RIPA = radioimmunoprecipitation assay

ROS = reactive oxygen species

SDS-PAGE= sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SNc = substantia nigra pars compacta

ST= striatum

TH = tyrosine hydroxylase

TNF-α = tumor necrosis factor alpha

TBS-T – tris buffered saline with tween-20

TLR = toll like receptor

WAVE2 = WASp family Verprolin-homologous protein-2

WT = wild type

Introduction

Overview

Parkinson's disease is the most common geriatric motoric neurodegenerative disease in the world, and yet its pathogenesis remains an enigma (Mhyre et al., 2015). Growing evidence implicates both environmental factors and the gut brain axis in the causality of PD (Klingelhoefer & Reichmann, 2015). Phage 936 has been found in above normal concentrations in the gut of patients with PD (Tetz, Brown, Hao &Tetz, 2018). It may constitute an environmental trigger, as it can be consumed in contaminated milk due to its ability to evade pasteurization (Madera, Monjard´ın & Sua´rez, 2004). The consumption of phage 936 can cause dysbiosis through the destruction of its probiotic host bacteria *L. lactis*. Adding to its potential pathogenicity is reports that phages can interact with eukaryotic cells (Van Belleghem et al., 2017). No study to our knowledge has examined how phages effect the CNS or looked at how phage 936 may act as an environmental toxin in mammals.

Parkinson's disease (PD)

PD was first recognized in 1817 by James Parkinson and is a progressive, chronic and complex neurodegenerative disease (Kwon, 2018; Rocha, De Miranda & Sanders, 2018). Today there is an estimated 7 – 10 million people affected worldwide; including, 1 in every 500 Canadians (UCB Canada, 2018). The known prevalence of PD is 1% of the population, but its prevalence increases with age (Tysnes & Storstein, 2017). In Canada the average age of symptom onset is 64.4 years with diagnosis at 66.2 years; however, the younger the individual at symptoms onset, the longer the delay in diagnosis (Wong, Gilmour & Ramage-Morin, 2015). Men are more likely

to get PD at 0.3% compared to women at 0.2% when residing at home, but 6.6% versus 4.0% ($p < 0.05$) if they reside in an institution (Wong, et al., 2015).

Genetic factors are only known to account for a rate of incidence of 5-10% (Tysnes & Storstein, 2017); although most cases of PD are idiopathic, they likely involve environmental factors or gene-environment interactions (Sampson et al., 2016). A definitive diagnosis for PD can only occur post- mortem, but there are several diagnostic tools available to aid in a differential diagnosis (Behari et al., 2011). Two widely used tests are the Hoehn and Yahr Stages of Motor Function Degeneration and the Unified Parkinson's Disease Rating Scale (UPDRS) to assess non-motor symptoms (Parkinson's Foundation, 2018).

Pathophysiology

The substantia nigra (SNc) is the primary area of neurodegeneration in PD. It projects into the basal ganglia (BG), of which the striatum (ST) is a main component. The basal ganglia is associated with motor control as well as affective and cognitive functions; its degeneration may account for the varied symptomology of PD (Bostan & Strick, 2018). Characteristic motor symptoms of PD; include but are not limited to, muscle rigidity, resting tremors and bradykinesia. Non-motor symptoms; include but are not limited to, thermodysregulation, olfactory dysfunction, cognitive dysfunction, impairment mood disorders, sleep disturbances and GI issues (Mulak & Bonaz, 2015; De Virgilio et al., 2016; Wong et al., 2015).

The loss of dopaminergic neurons in the SNc and the accumulation of Lewy bodies in the CNS are hallmarks of PD (Tysnes & Storstein, 2017). Lewis bodies are insoluble, intracellular inclusion within neurons and glial cells; they contain aggregates of misfolded alpha-synuclein (Hoenen et al., 2016; De Virgilio et al., 2016). Alpha-synuclein comprises 1% of the brain's

cytosolic proteins but are most highly expressed in the dopaminergic neurons of the SNc (Hoenen et al., 2016). Alpha-synuclein maybe responsible for neurotransmitter release, plasticity, synaptic function and the trafficking of synaptic vesicles (Rocha, De Miranda & Sanders, 2018; Hoenen et al., 2016). Aberrant alpha-synuclein can cause dopaminergic cell death by binding to the lipids and disrupting cell membranes, as well as by activating microglia directly (Hoenen et al., 2016). Dopamine is a key regulator of cytokine secretion in the adaptive and innate immune response; for example, it suppresses TNF-alpha and promotes CXCL1 (Kawano, Takagi, Saika, Matsui & Matsushita, 2018).

Both peripheral and central inflammatory stressors contribute to the neurodegeneration in PD (Calabresea et al., 2018). The brain is considered immune-privileged due to the blood brain barrier (BBB), low major histocompatibility complex (MHC) class I and II expression and absence of antigen presenting cells in brain parenchyma (Louvea, Harris & Kipnis, 2015). It is; however, not completely isolated from peripheral inflammation. The dura matter layer of the meninges contains a lymphatic system which allows immune cells to drain from the CNS into the peripheral lymphatic system via deep cervical lymph nodes (DCLN) (Louveau, Harris & Kipnis, 2015; Thomas, 2017; Kipnis & Filiano, 2017). The meningeal lymphatic endothelial cells may also directly influence adaptive immunity by regulating tolerance to self-antigens in T-cells; inflammation could compromise this interaction (Louveau, Harris & Kipnis, 2015). The SNc in PD has infiltration of peripheral $CD4^+$ and $CD8^+$ T-lymphocytes and a reduction in the suppression of regulator T-cells by effector T-cells (Calabresea et al., 2018).

Microglia are the first line, innate immune effector cells of the brain (Keren- Shaul, 2017). Their activation is region dependent and heterogenous (Grabert et al., 2016; Sampson et al., 2016). Some phenotypes beneficially release anti-inflammatory cytokines and neurotrophic

factors, while other noxious phenotypes release pro-inflammatory cytokines, ROS and proteinases (Beltran-Castillo, Eugenin & Von Bernhard, 2018).

Microglia have several phenotypes; in a resting state their morphology is ramified with many fine processes in which to scan their environment (Olah, Biber & Boddeke, 2011). Microglia contain unique transcripts for proteins that can sense microbes and endogenous ligands, these are referred to as sensomes (Hickman et al., 2013). As microglial sense their environment with sensomes, they change their phenotypes in accordance (Grabert 2016). Once activated they morphologically changes based on the stressor; under inflammatory conditions the microglia become amoeboid while under chronic stress and aging they become hyper-ramified (Dubbelaar et al., 2018). Several intermediate morphologies also occur. Phenotypes differ not only by shape but also by composition; IBA-1 positive microglia are comprised of 94% synaptic elements, including dendritic spines, axon terminals, synaptic clefts and astrocytic processes (Bisht et al., 2016). Bisht et al. (2016) identified a microglial phenotype, “dark microglia”, with condensed nucleoplasm and cytoplasm, chromatin remodeling and dilated endoplasmic reticulum. Dilated endoplasmic reticulum is indicative of increased oxidative stress (Brisht, et al., 2016).

Dark microglia are phagocytically hyperactive; engulfing entire synapses, axon terminals and dendritic spines. This phenotype was rare in healthy controls but found in abundance in normal aged, chronic stressed, AD and CXCR1 knock out mice. Additionally, Keren- Shaul et al. (2017) found populations of disease-associated microglia (DAM) in aged individuals as well as in those with neurodegenerative diseases (Kipnis & Filiano, 2018). DAM have pro-inflammatory activity and are associated with disease acceleration (Keren-Shaul et al., 2017).

Microglia moderate the inflammatory response in the brain; neuroinflammation maybe the key to the development of PD (Spittau, 2017; Salter & Stevens, 2016). Inflammation causes oxidative stress and cytokine-dependent toxicity, which may contribute to the degeneration of the nigrostriatal pathway in sporadic PD (Tansey & Goldberg, 2009). The microglial inflammatory phenotype is characterized by increased pro-inflammatory cytokine, major histocompatibility complex II (MHCII), toll- like receptors (TLR) and inflammatory receptors (Han, Harris & Zhang, 2017; Soreq, Rose, Soreq & Hardy, 2017; Spittau, 2017). This is supported by autopsy results. Those with PD showed activated microglia, Nuclear Factor kappa B (NFkB) pathway activation, accumulation of cytokines as well as oxidative damage to protein in the brains and cerebral spinal fluid (CSF) (Tansey & Goldberg, 2009).

Aging

Although poorly understood, age is the greatest risk factor for the neuroinflammation and neurodegeneration that occurs in PD (Tansey &Goldberg, 2009). PD may represent a type of segmental aging, in which dopaminergic neurons (DA) in the pars compacta of the SNc are specifically affected (Khojah, Payne, McGuinness & Shiels, 2016; Calabresea et al., 2018). Aging is also associated with altered B and T cell capacity, a reduction in the number of intestinal Peyer's patches and a state of chronic macrophage activation (Garcia-Pena, Alvarez-Cisneros, Quiroz-Baez & Friedland, 2017). "Inflammaging" is a term adapted to characterize the hormetic or hormetic- like effect of low-grade, chronic, systemic inflammation associated with aging (Franceschi & Campisi, 2014; Calabresea et al., 2018). The microglia become primed and can undergo epigenetic modifications with potential pathogenic gene expression (Scheffold 2016; Spittau, 2017; Frank, Weber, Watkins & Maier, 2015). Over time divergent cellular

events, such as protein misfolding, mutations and aggregations, may cause regional microglial activation. This can lead to a self-sustaining cascade of neuroinflammation (Grabert et al., 2016; Tansey & Goldberg, 2009).

Aging is also associated with a reduction in mitochondrial efficacy and mitophagy which results in an accumulation of reactive oxygen species (ROS), damage to proteins and DNA, a reduced number of mitochondria and less ATP production (Calabresea et al., 2018). An increase in oxidative stress and mitochondrial dysfunction is also associated with PD (Calabresea et al., 2018). Even under non-stimulated conditions pro-inflammatory cytokines and ROS were elevated with age (Beltran-Castillo, Eugenin & Von Bernhard, 2018).

The microglia in aged animals have increased senescence and dysfunction, which has been linked to telomere shortening and lysosomal inclusions caused by an increase in myelin fragments (Spittau, 2017; Scheffold et al., 2016). These microglia have impaired regulation, as activation is amplified and prolonged, resulting in an increase of inflammatory factors such as nitric oxide (NO) and TNFα (Norden & Godbout, 2013; Spittau, 2017). Dubbelaar et al. (2018) used genetically altered mice (deficient in DNA repair protein Ercc1) as an aged model. They found hyper-ramified microglia and an upregulation in gene expression for; phagocytosis (Cle7a and Axl), interferon, pro-inflammatory cytokines (IL-1B, IL-6 and TNF- alpha), vesicle release, proliferation and zinc binding. These mice also had increased ROS and lymphocyte activation, with different brain regions showing divergent levels of aging.

Gut- brain axis

There is a reciprocity between gut microbiota and neurological health; 80% of those with PD have gastrointestinal (GI) dysfunction, which in turn causes pathological exacerbation

(Mulak & Bonaz, 2015). This relationship is enhanced by the vagus nerve, which both stimulates the gut and allows aberrant alpha-synuclein to travels from the gut to the brain (Braak's hypothesis); a vagotomy had been shown to reduce the rate of incidence of PD in patients (Mulak & Bonaz, 2015; Kujawska & Jodynis-Liebert, 2018; Liu et al., 2017). GI issues such as inflammation, decreased motility and constipation, precede motor deficits by several years (Sampson et al., 2016). Constipation occurs is 87% of PD patients and is considered a harbinger for the disease (Perez-Pardo et al., 2017).

PD progresses in predictable stages; initiating in the gut and proceeding to the brain (Kwon, 2018). Aberrant alpha-synuclein accumulates and causes damage to neurons within the enteric nervous system (ENS) (Perez -Pardo et al., 2017). The ENS is an integrated network of neurons within the GI wall and when damaged may cause increased intestinal permeability "leaky gut" (Perez -Pardo et al., 2017). TLR2 was found to regulate the integrity of the intestinal barrier as well as have an association with neuroinflammation in PD (Mulak & Bonaz, 2015). Leaky gut could initiate systemic inflammation due to the translocation of gut microbiota; including, bacteriophages (phage) and gut bacterial such as *Escherichia coli (E. coli)* (Perez -Pardo et al., 2017). *E. coli* is a gram-negative bacterium which contains the endotoxin lipopolysaccharide (LPS). LPS binds to TLR-4 on microglia and elicits a strong immune response; including, initiating the translocation of NFkB to the nucleus for the transcription of pro-inflammatory genes such as COX-2 and IL-8 (Petsel, et al., 2017). It further induces the production of various immunoregulators, proinflammatory cytokines, free radicals and a deficit in fractalkine receptor CX3CR1, which collectively damage DA neurons in SNc (Wang, Liu & Zhou, 2015). Platelet-activating factor (PAF) and related phospholipid products (OxPls) are also made in response to LPS, which further increases inflammation (Petsel, et al., 2017).

Gut microbiota can further upregulate both local and systemic inflammation caused by LPS (Mulak & Bonaz, 2015). Leaky gut is a trigger for chronic inflammation, which underlies the development of many diseases including PD (Tetz & Tetz, 2018). Furthermore, the peripheral immune response of pro-inflammatory cytokines (such as TNF-alpha, IL-B and IL-8) in the serum may induce BBB disruption thus promoting microglial activation and neurotoxicity (Mulak & Bonaz, 2015).

The pathogenesis of PD can be exacerbated by dysbiosis or microbial imbalance within the gut, this can have far reaching consequences, including peripheral and central inflammation (Perez-Pardo, 2017; Bell et al., 2018). The composition of host microbiota affects microglial regulation, maturation and function; age may also be a contributing factor as it is characterized by lower species diversity (Erny et al., 2015; Mulak & Bonaz, 2015). Sampson at al. (2016) found that a fecal transplant from PD patients to mice that over-expressed alpha-synuclein caused the mice to exhibit motor dysfunction; indicating gut microbiota was necessary for motor deficits as well as alpha-synuclein pathology and microglia activation. Symbiotic microbiota modulate several complex processes in order to maintain systemic homeostasis; such as, metabolism, immune response, neurotransmission, electrophysical activity within the ENS and energy balance (De-Paula, Forlenza & Forlenza, 2018; Sarkar et al., 2016).

Based on studies using germ -free mice, microbiota also help decrease the permeability of the BBB by producing metabolites that strengthen endothelial tight-junctions (Sun & Shen, 2018). Gut microbes differ between healthy and PD individuals, potentially leading to alterations in the production of neurotransmitters, hormones, neuropeptides and immune signals (Sampson et al., 2016; De-Paula, Forlenza & Forlenza, 2018). A decrease in the total count of intestinal bacteria predicted an increase in UPDRS score (increase in PD progression) (Minato et al.,2017).

The gut microbiota of individuals with PD yielded a 10x reduction of the dopamine producing *Lactococcus*, with an increase in the lytic *Lactococcal* bacteriophages (phage) 936 (Tetz, Brown, Hao &Tetz, 2018). *Lactococcus lactis* is a symbiotic bacterium probiotic (Stoyanova et al., 2017). Probiotics are defined by the World Health Organization and the Food and Agriculture Organization of the United Nations (2001), as live microorganisms that have health benefits when administered in adequate quantities (Stoyanova et al., 2017). Probiotics have been shown to downregulate the hypothalamic- pituitary-adrenal (HPA) axis and promote GABA, which is reduced in patients with depression (Ng et al., 2018; Sarkar et al., 2016). Probiotics represents "microbial organs" which produce and respond to host neurotransmitters, hormones and neuromodulators; directly and indirectly alter host's mood, behavior, sleep, appetite, metabolism and cognition (Stoyanova et al., 2017; Kali, 2015).

Phage as a human pathogen

Phages are viruses that specifically infect host bacteria. They are the most abundant organism and virus in the biosphere and outnumber all other organisms in the human body (Abedon, 2016; Clokie, Millard, Letarov, & Heapy, 2011; Nguyen et al., 2017). Phages have been generally regarded as having symbiotic relationship within the gut microbiota; however, a new study by Tetz et al. (2018) has identified them as a potential human pathogen (De paepe et al., 2014; Navarro & Muniesa, 2017). Phages are able to genetically alter the bacteria within the body (Navarro & Muniesa, 2017). Their capsid contains genetic material, which once injected into the host bacterium results in viral gene expression and changes in the host cell's metabolism (Abedon, 2016). There are two main types of phages, lytic and lysogenic. The lytic type replicates within the host bacteria cell and release virions, thereby killing the host (Tetz & Tez,

2018). The lysogenic type passively replicates within the host, after it incorporates its genetic material into the host's genome and creates prophages; phage- encoded toxins can be released by this type (Tetz & Tetz, 2018; De Paepe, Leclerc, Tinsley & Petit, 2014). Lysogenic phages can be induced into the lytic state with oxidative stress, DNA damage and temperature (De Paepe et al., 2014). Phages' capacity for horizonal gene transfer and their potentiation of bacterial evolution have led to the belief that they are key to understanding microbial systems (Clokie, et al., 2011). The impact of phages on the human body; however, has been largely overlooked (Nguyen et al., 2017; Navarro & Muniesa, 2017). Even the lysogenic type phage can contribute to pathogenesis, by genetically altering bacteria and causing dysbiosis (Tetz & Tetz, 2018). Furthermore, during viral infection bacteria produce metabolites, short-chain fatty acids, that cause microglial activation as well as alpha-synuclein aggregation (Sampson et al., 2016).

Despite phages' ability to disseminate throughout the human body and bypass endothelial tissue layers, the precise mechanism of action is not known; however, previous research has implicated; phagocytosis, clathrin-mediated endocytosis, micropinocytosis, caveolae- mediated endocytosis, non-specific endocytosis, Golgi-mediated route, M cell -mediated pathways in gut, cellular penetrating peptides, various ligand displays and integrins (Nguyen et al., 2017; Huh, Wong & St. Jean, 2019). Nguyen et al., (2017) suggest that 31 billion phages cross the gut's epithelial layers in a human every day. Tetz & Tetz (2016) administered a phage cocktail to rats, which resulted in leaky gut and a subsequent increase in pro-inflammatory cytokines and plasma endotoxins. As phages destroy their host bacteria, peptidoglycans bacterial amyloids, cell -free DNA and other cellular debris pass through the permeable intestinal wall, into the circulation. The bacteria debris is recognized as PAMPs and activates innate immune response (Tetz & Tetz, 2018). Viral nucleic acids are also recognized as PAMPs by several TLRs (TLR3, TLR7, TLR8

and TLR9) which induces the production of type I IFN and other inflammatory cytokines (De Paepe et al., 2014). Phages can enter areas of the body that are considered sterile. It was believed that the presence of bacteria and viruses in the CSF would lead to meningitis. Tetz & Tetz (2018) suggest that the presents of phages in the CSF may cause or contribute to neurodegenerative diseases. The consequences of phages may have gone unnoticed due to human fluids analysis traditionally being done with 16s RNA gene sequencing rather than the shotgun sequencing required to identify phages (Tetz & Tetz, 2018).

Phages can bypass the BBB, which only allows highly lipidized molecules less than 400 Daltons to pass through; due to endothelial tight junctions, astrocyte end-feet processes, pericytes and perivascular neurons (Nathanson & Mischel, 2011). Phages may target certain ligand motifs and clone their phage coats to gain access (Nathanson & Mischel, 2011). Staquicini et al. (2011) found that phages can utilize the transferrin and transferrin receptor (TfR) system, which normally enables peptides to cross the BBB by undergoing conformational changes when iron binds to transferrin. Phages can display an iron-mimic peptide to access the TfR system and gain access through the BBB and thus infect the brain (Staquicini et al., 2011).

Phages where found to contain prion like domains and be associated with protein misfolds (Tetz et al., 2018; Tetz & Tetz, 2017). Prions are alternatively folded, self -replicating proteins, that potentially cause disease (Prusiner et al., 2015). Prion domains within phages could potentially interact with eukaryotic proteins, and cause prion-like protein misfolding in humans; aberrant alpha-synuclein is considered a prion (Tetz & Tetz, 2017; Prusiner et al., 2015).

To date no studies have been published that examine whether or not phages cause neuroinflammation. PD is disease with multifaceted causalities based largely on the

neurodegenerative effects of neuroinflammation. It is suggested that the inflammatory response is altered with age, due to such factors as microglial senescence and priming. This study examines phage 936, found in dairy products, in relation to age and LPS as a potential cause of neuroinflammation.

Dietary phage exposure and PD

Dairy products are a source of both the bacteria *Lactococcus lactis* as well as its host specific virulent phage 936; phage 936 is a member of the Siphoviridae family and is characterized by a long non-contractile tail, short latent period and a high burst size (Chmielewska-Jeznach, Bardowski & Szczepankowska, 2018). The dairy industry represents the largest human controlled bacterial cultures, and is highly susceptible to bacteriophage infection (Chopin, Chopin & Bidnenko, 2005). Several studies have reported that consuming large amounts of dairy increases the risk of PD. Jiang, Chuanxia, Jiang and Zhang, (2014) used data from seven studies totaling 1,083 PD cases among 304,193 subjects. They found a 17% increase in the incidence of PD for every 200g/day for milk and an increase of 13% for every 10g/day of cheese. Likewise, Chen et al. (2007) used the American Cancer Society's Cancer Prevention Study II Nutrition with a cohort of 57,689 men and 73,175 women. 250 men and 138 women were identified as having PD nine years later. The relative risk of acquiring PD was found to be 1.8 for men and 1.3 for women who consume high amounts of dairy. Additionally, Hughes et al. (2017) used Nurse's Health study with a cohort of 80, 736 and Health Professionals Follow-up study with a cohort of 48,610 with respective 26 and 24 year follows and found that low fat milk consumption of three servings a day increased the hazard ratio by 1.39 compared to those who consumed none. Another study utilized data from the EPIC-Greece population-based cohort of

28, 572 volunteers showed a significant health risk of 1.34 when milk was consumed daily (Kyrozis et al., 2013). Further studies by Abbott et al. (2016) and Parks et al. (2005) used data obtained from Honolulu-Asia aging study; milk intake data was collected from 1965 to 1968, on 449 men aged 45-68 years, with post-mortem examinations from 1992 to 2004. Abbott et al. (2016) found that neuronal density what significantly lower and the risk for PD significantly higher, for those who consumer more than 16oz of milk per day. Parks et al. (2005) found that there was a 2.3-fold increase in the incidence of PD in those who consumer more than 16oz of milk per day.

Bacteriophages found in dairy products may contribute to the increased PD risk (Tetz et al., 2018). Some phages are more susceptible to pasteurization; for example, phage 936 is 35 times more heat resistant than phage c2 (most abundant phage found in raw milk) (Madera et al., 2004). Whey samples showed 20 of 24 (85%) still contained 936 phages, while c2 was detected in only one (4%) of these samples (Madera et al., 2004). Further studies have found that *Lactococcus lactis* phage 936 has a heat stabilizing genetic factor CB14 which allows it to remain a major risk in milk fermentation processes (Geagea, Labrie, Subirad, & Moineauc, 2018). Phage 936 has proven to be difficult to control in the dairy industry and continues to dominate as the single most problematic phage species in the industry (Mahony, Murphy & Sinderen, 2012).

Thesis Aims

The present thesis sought to assess the impact of phage 936 in young or old mice and whether the virus augments or diminishes the impact of an inflammatory (LPS) stimulus. To this

end, an initial study was conducted to first determine if the bacteriophage was present in the brain and intestine following ingestion and if it alone could actually produce some degree of measurable changes (e.g. neutrophil mobilization or change in cytokine or other circulating immune factors) within the brain. The main study of this thesis involved young (4-5 months) vs old (15-16 months) mice receiving the phage 936 (or vehicle), followed by LPS (or vehicle) treatment. We assessed peripheral gut and brain inflammatory changes, as well as assessed motor functioning and sickness along with dopamine neuronal counts. We hypothesize that the old mice that receive both the LPS and phage 936 would display the greatest degree of inflammatory and neuronal pathology. However, was also possible that the bacteriophage would diminish the impact of LPS given that phages can neutralize endogenous bacteria and hence, might limit the inflammatory profile. Whatever the case, novel potentially very important data relevant for inflammatory aging in general and PD in particular, was gathered since a study of this nature, to our knowledge, has never been conducted.

Aim 1

To determine if the phage alone will produce some degree of measurable changes (e.g. neutrophil mobilization or change in cytokine or other circulating immune factors) within the brain.

Aim 2

To determine if pathologies (brain inflammatory changes, reduction in dopaminergic neurons, decline in motor function or increase in sickness score) produced by the phage will be altered by LPS or age.

Hypothesis

We hypothesize that both LPS and age will exacerbate the immunological changes produced by the phage, thus producing detectable pathology in the mice.

Methods

Initial Study

Mice were trained for seven days to eat pudding (as a means for delivering the phage); this involved placing pudding on a plastic dish in the mouse's cage and repeating every 24hrs. Mice (n=3/group) were randomly assigned to one of three experimental conditions (pudding no phage; pudding with phage once a day and pudding with phage twice a day). The mice were maintained on their regular diet of Harlan mouse chow and water *ad libitum*. All experimental procedures were approved by the Carleton University Committee for Animal Care, which complied with all guidelines set out by the Canadian Council for the Use and Care of Animals in Research.

One microlitre of phage 936 was added to 0.1 ml of pudding and fed to mice for 12 days (Tetz &Tetz, 2016). The mice were sacrificed by rapid decapitation on the 13th day. Brain tissue, upper section of intestine and fecal pellet was collected and flash frozen until assayed. The SNc, ST, prefrontal cortex (PFC), hippocampus and intestine underwent polymerase chain reaction (PCR) to determine presence of phage.

Phage 936 replication and purification.

Medium M17 (0.5% lactulose) was inoculated with host bacteria *Lactococcus lactis* (University of Laval, Montreal, QC) and incubated overnight at 30°C. Phage 936 (1x 10^6 pfu/ml (University of Laval, Montreal, QC) was added to the bacteria the next day. The phage lysate of the second phage propagation was added to obtain 1x10^5 pfu/ml and incubated for five hours to yield titres 1x10 pfu/ml. The phage was purified by centrifuging at 10,000rpm for 15 min at 4°C. NaCl and PEG800 was added to the supernatant; the solution was incubated overnight at 4°C. The solution was centrifuged as before, and the pellet was resuspended in phage buffer. The resuspended phage was placed in a 15 ml conical tube. Three solutions of CsCl in phage buffer was used to create a gradient; solution A 4.2g/8ml, 6.2g/8ml, 8.2g/8ml. The phage was then added to the top of gradient to fill the tubes. The tubes were placed in swinging buckets, placed on the rotor and then centrifuge at 35,000rpm for three hours at 20ºC. The extracted phage was centrifuged again at 40,000 rpm for 18 hours at 20ºC. The phage was extracted, through the side of the tube, as before. The extraction was dialysed (3x20min) against phage buffer, using a dialysis cassette. The purified phage was stored at 4ºC in a screw cap tube.

PCR.

DNA extraction was performed using the DNeasy Blood & Tissue Kit (Qiagen, MD) and provided protocol. The DNA primer, determined by Labrie and Moineau (2000) for phage 936, was used to detect the presence of phage in the tissue. The DNA was amplified using PTC-200 thermocycler (MJ Research).

Primary study

Animals and general experimental design.

The timeline of the experiment is shown in **Figure 1**. A total of 96, three and a half - month-old, C57BL6 mice from Charles River Laboratories (Montreal, QC, Canada) were used. Forty-eight of these mice arrived 15 and a half months prior to the commencement of experimentation and were aged in the vivarium. The mice will be fed a regular diet standard diet (Harlan, 2014) mouse chow, water *ad libitum*, and room temperature was maintained at ~ 21 °C. The mice were housed 4 to a standard polypropylene cage (27 × 21 × 14 cm) and given an enriched environment of nestlet and a house. The other 48 mice will arrive two weeks prior to experimentation so that they can acclimate. These mice were housed in the vivarium in the same manner as previously described.

The general experimental design is a N=96 with n=48 old and n=48 young. These mice will be randomly selected to receive pudding with or without the added phage (n=24/group). The mice were further randomly selected to receive two doses of 1 mg/kg LPS or saline (n=12/group). Three days prior to experimentation the animals were trained on pudding administration. The mice receive 0. 1 ml of chocolate pudding (either with or without phage at a concentration of $1x10^7$ PFU) for 12 days. The last two days (day 11 and 12) were concurrent with the LPS administration. The second dose of LPS was given 24 hours after the first dose (both IP at 1mg/kg). Animals were sacrificed 24 hours after the second LPS injection. Two relatively modest LPS doses were utilized in order to minimize lethality that sometimes becomes

apparent in aged mice. Although modest, these two doses should be sufficient to induce obvious sickness and inflammatory responses (e.g. including cytokine and microglial changes).

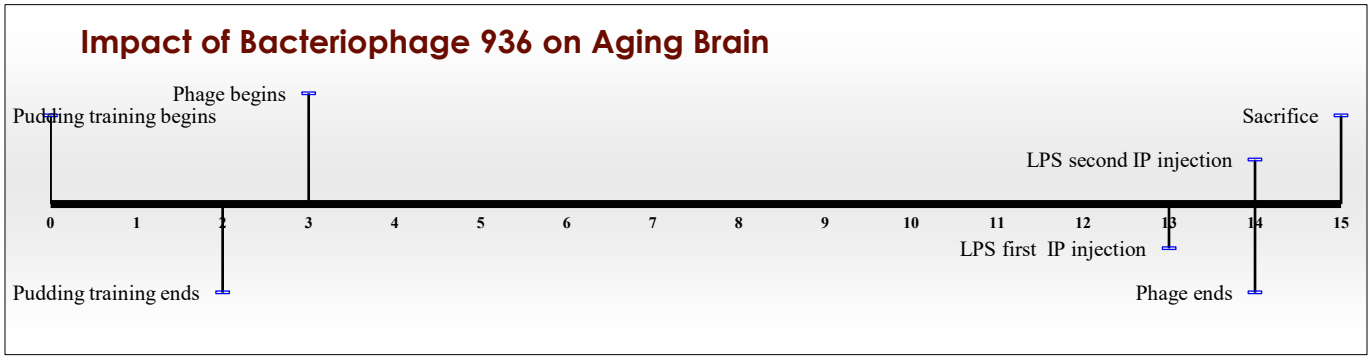


Figure 1: Timeline. Pudding training occurred for three days followed by phage administration for 12 day. On the last two days of phage administration LPS was given concurrently IP. After 12 days of phage and 2 days of LPS the mice were sacrificed 24hrs after the last LPS injection

Immunofluorescent staining.

Slices of the SNc were stained for tyrosine hydroxylase (TH) to identify dopamine neurons, as well as ionized calcium binding adaptor molecule 1 (IBA1) and CD68 to identify phagocytic cells. TH is found in dopaminergic neurons and is a catecholamine biosynthesis rate-limiting enzyme (Daubner, Le & Wang, 2011). IBA1 is a membrane bound protein in macrophages which is upregulated in an active state (Ohsawa, Imai, Sasaki & Kohsaka, 2004). Brains will be flash frozen and sliced in the Fisher cryostat on Shandon AS620 cryostat (Fisher Scientific, Ottawa, ON) at a thickness of 40 µm to optimize the penetration of antibodies. The SNc was sliced and every third slice was used for staining. Sections were washed in PBS (pH 7.2) (3x5min) then placed in the blocking solution (5% NGS, 0.3% triton-X and 94.7%PBS (pH 7.2)) for 1hr. Slices were placed in the primary solution (5% NGS, 0.3% Triton X, 0.3% BSA, 94.4% PBS) with their respective antibody for 2hrs; mouse TH (1:2000), rabbit IBA1 (1:2000);

rat cd68 (1:2000). Slices were washed in PBS (3x5min) and placed in the secondary solution (5% NGS, 0.3% Triton X, 0.3% BSA, 94.4% PBS) with the appropriate antibody of anti-mouse 488 (1:1000), or anti-rabbit 647 (1:1000) respectively. Slices were mounted on slides and slip covered using fluoromount. Slides where scored based on the scale presented by Colburn et al., (1997): one for extensive ramification, wispy, well spaced, undisturbed; two for less ramified, less well spaced and signs of immunoreactivity; three for increasingly less ramified, occasional increased density, maybe some overlap, prominent immunoreactivity and four for short bold projections, dense with extensive overlapping and intense immunoreactivity. Images were viewed using immunofluorescence microscopy using Zeiss image acquisition software (Zeiss LSM 510).

Behavior and sickness score.

Prior to sacrifice all mice were assessed for two minutes for signs of sickness. A Likert scale was used with scoring based on; one for normal appearance, two for ptosis, lethargy or piloerection and three for destressed, curled body posture and altered responsiveness (Hayley et al., 1999).

Sacrifice.

Half of each cohort was injected with sodium pentobarbital and transcardially perfused so that tissue could be collected for immunostaining. The remaining mice were sacrificed through rapid decapitation and tissue was collected for Western blot analyses. Briefly, for Western blot, animals were rapidly decapitated with surgical scissors at the cervical vertebrae. Regions of the ST, SNc, prefrontal cortex and hippocampus were excised by micro-punch from coronal brain

sections using a chilled micro-dissecting block (0.5mm slots) for use in Westerns. The tissue was placed in vials of PB on ice. For immunohistochemistry, tissue was fixed by flushing out the blood with 5ml of saline followed by 40ml of 4% paraformaldehyde via a needle in the right ventricle of the heart. The brain and intestine were extracted and placed in vial of 4% paraformaldehyde then placed on ice. The brains was stored in the refrigerator at -20 º C. The brains were then transferred to a vial containing 10% sucrose 0.1M PB solution (pH 7.4) after 24hrs. After an additional 24hrs the brains were transferred to 30% sucrose 0.1M PB solution.

Tissue collection.

The intestine was collected for future studies, it is relevant since phages are found at a 4x higher concentration in the mucus layer compared to the lumen within the gut of mice (Barr et al., 2013). The intestines are stored in the -80 º C. One fecal pellet per mouse was collected at time points of baseline (prior to phage administration) and prior to sacrifice. DNA was extracted from the pellet using the MagMAX Microbiome Ultra Nucleic Acid Isolation Kit to extract the bacterial DNA which was subsequently sent away for analysis. Blood will be drawn prior to sacrifice and stored in tubes in the -80 º C.

Plasma lipocalin assay.

Neutrophils release large amount of LCN2 upon activation; therefor is a good indicator of peripheral inflammation (Shao et al., 2016). A plasma lipocalin assay was performed to quantify the circulating LCN2 in the mice. First the mice were decapitated and the blood from the trunk was collected and placed into 10 μg EDTA. Then the blood samples were centrifuged (2000g for 20 min), subsequently the plasma was extracted and then stored in aliquots at −80 °C for later

ELISA. The commercially available ELISA kit (Invitrogen, USA, Cat #EMLCN2) was used following manufacturer's instructions. Each sample was assayed in duplicate, in a single run to control for inter-assay variability; the intra-assay variability was less than 10%.

Western blot.

Based on previous age-related studies from the Hayley lab the following biological protein markers where assessed; GFAP, CX3CR1, WAVE2, Sirtuin 3 (Sirt3), MFG-E8 and Caspase 1 (Cas1). A) Glial fibrillary acidic protein (GFAP) is an indicator of astrocytes activation. (b) CX3CR1 is an indicator of the level of immune activation; injured neurons release the chemokine domain of fractalkine which attracts microglia and triggers their activation. The receptor ligand pair CX3CLL1-CX3CR1 acts as a check point for the inflammatory response, directly inhibits microglia interaction with neurons (Keren-Shaul et al., 2017). (c) WAVE2 is a cytoskeleton regulatory protein which may play a key role in inflammation. Microglia undergo morphological changes from ramified to amoeboid (Erny et al., 2015). Senescence changes neuroinflammatory responses, threaten aged neurons and increases age-related neurodegenerative pathologies (Spittau, 2017). (d) Sirt3 is an intracellular regulatory protein (Genecard, 2018). It is a NAD^+-dependent deacetylase and has a role in metabolism regulation and energy balance, associated with longevity (Kim, Cheresh, Jablonski & Kamp, 2015). It is important in regulation of ROS and mice deficient in Sirt3 have hypersusceptible to colonic inflammation and tumor development (Zhang et al., 2018) (e) MFG-E8 is a bridging protein that mediates antigen presentation and may be involved in the phagocytosis of neurons by microglia (Neniskyte & Brown, 2013). (f) Cas1 is a marker for inflammation and infection (Bauernfeind & Hornung, 2013; Skeldon & Saleh,2011).

Protein extraction.

Tissue punches from the SNc and ST were collected. The whole cell lysates from the brain regions of interest (ST& SNc) was homogenized RIPA buffer (50 mM Tris (pH 8.0), 150 mM sodium chloride, 0.1% SDS, 0.5% sodium deoxycholate and 1% Triton X-100) with Complete Mini EDTA-free protease inhibitor (Roche Diagnostics, Laval, QC) followed by two seconds of sonication. The lysed cells were centrifuged at 6000RPM for 10 minutes at 4˚C with a tabletop microcentrifuge (MBI Prism R). The supernatant was extracted and bicinchoninic acid (BCA) method (Thermo Scientific) was used to determine samples' protein concentration. Samples were quantified using Soft Max software and SpectroMax 190.

Proteins were denatured by placing 5X loading buffer (containing 5% glycerol, 5% β-mercaptoethanol, 3% SDS and 0.05% bromophenol blue) into the samples, followed by heating them at 100 ˚C in a heating block for five minutes. The samples were stored in the freezer at -20˚C.

SDS-PAGE electrophoresis.

A gel comprised of 10% separating buffer (370 mM Tris-base (pH 8.8), 3.5 mM SDS) and stacking buffer (124 mM Tris-base (pH 6.8), 3.5 mM SDS) was placed in running buffer (25 mM Tris-base, 190 mM glycine, 3.5 mM SDS). Protein samples were removed from the -20˚C freezer and placed on ice. The ladder and samples were loaded into the Acrylamide gel (10%) and run for one hour at 140 volts.

Protein transfer.

The proteins were transferred from the gel to a PVDF membrane (Bio-rad) for one hour at 4 ˚C at 100 volts in transfer buffer (25 mM Tris-base, 192 mM Glycine, 20% methanol). Membranes were dried then rinsed with methanol. To determine total protein concentration, the membrane was incubated in REVERT and then placed in a REVERT wash solution (6.7% Glacial Acetic Acid, 30% Methanol, in water). The LI-COR Odyssey imaging system, set at 700 channel for two minutes will be used to acquire and image. The membranes were rinsed in TBS (pH 7.5) buffer and blocked in 0.5% fish gelatin (Sigma) in TBS. After 90 minutes the membranes were placed in primary solution (0.05% fish gelatin in TBS, 0.1% tween, and antibodies: rabbit anti-GP91 (1:5000)). After one hour the membranes were rinsed with TBS-T (3x) followed by an hour incubation in the dark with the secondary solution (0.5% fish gelatin, 0.2% tween, 0.01% SDS, infrared conjugate directly against the species used for the primary antibody (mouse, rabbit; 800 LICOR)). The membrane was washed in TBST-T (3x 5 minutes) then TBS (3x 5minute) and read on the LI-COR Odyssy system at 800 channel for six minutes and 700 channel for two minutes.

Statistical analyses.

The data on protein concentration, obtained through Western blots, was analysed using 2 x 3 ANOVAs (phage x LPS x age). In addition, the Fisher post-hoc test ($\alpha = 0.05$) using statistical software StatView (version 6.0), SPSS (statistics 25), GraphPad Prism 5 were used where appropriate, with differences being considered significant at $p < 0.05$.

Results

Behavioral changes in sickness score and weight loss

Behavioral changes in 4-5 month-old (classified as young) and 15-16 month-old (classified as old) C57BL6 mice were assessed using a survival score, composite sickness rating scale and by measuring weight loss after the administration of phage 936 (10ul/ml) (or control) orally in pudding and/or LPS (1mg/kg) (or vehicle). (**Figure 2**). Comparison of survival curves showed significance (Mantel-Cox Chi square 14.52 p=0.0427 with Logrank test for trend Chi square 5.098 p=0.024). Treatment groups had a 100% survival rate, with the exception of one mouse in the old, phage, LPS (OPL) treatment group and three mice in the old, no phage, LPS (ONL) group, which did not survive to the experimental end, (ie. dying shortly after the second LPS injection) (**Figure 2A**).

After the first LPS injection old mice were significantly sicker than Young mice ($F(1,83)$, 18.19 $p< 0.0001$) and LPS mice were sicker than saline mice ($F(1,83)$ 92.04, $p<0.0001$) (**Figure 2B**). In addition, there was a significant Age x Treatment interaction in sickness ratings $F(1,83)$ 18.91 $p<0.0001$(**Figure 2B**). The follow up comparisons revealed that LPS treatment significantly elevated sickness ratings, but this effect was especially pronounced in the aged mice (**Figure 2B**). There also was a trend towards a Phage main effect with regards to sickness ratings that just missed significance $F(1,83)$ 3.78 $p=0.055$. In this case, there was a modest tendency for the phage treatment to elevate signs of illness (especially in the older mice).

After the second LPS injection, similar results were observed, with a significant increase in sickness score in Old mice ($F(1,83)$ 4.12, $p=0.046$), LPS mice ($F(1,83)$ 71.53, $p<.0001$) and Age x Treatment interaction $F(1,83)$ 4.12 $p=0.046$; in the absence of any effect of the phage

treatment. Between group comparisons revealed a pattern of effects strikingly similar to what was evident after the initial LPS injection. Specifically, the LPS increased sickness ratings in all animals, but those in the "old" cohort were most dramatic, such that they exceeded that of the younger mice ($p < 0.05$; **Figure 2 C**).

Paralleling the emergence of sickness, variations in weight loss were evident after both the first and second LPS injections ($F(1,83)$ 348.299, $p<0.0001$) and ($F(1,83)$ 61.658, $p<0.0001$), respectively. Indeed, the LPS treatments caused a dramatic loss of weight on both occasions, relative to non-LPS treated mice ($p < 0.05$) (**Figure 2 D and E**). In contrast, the phage and age variables did not significantly modulate this weight loss.

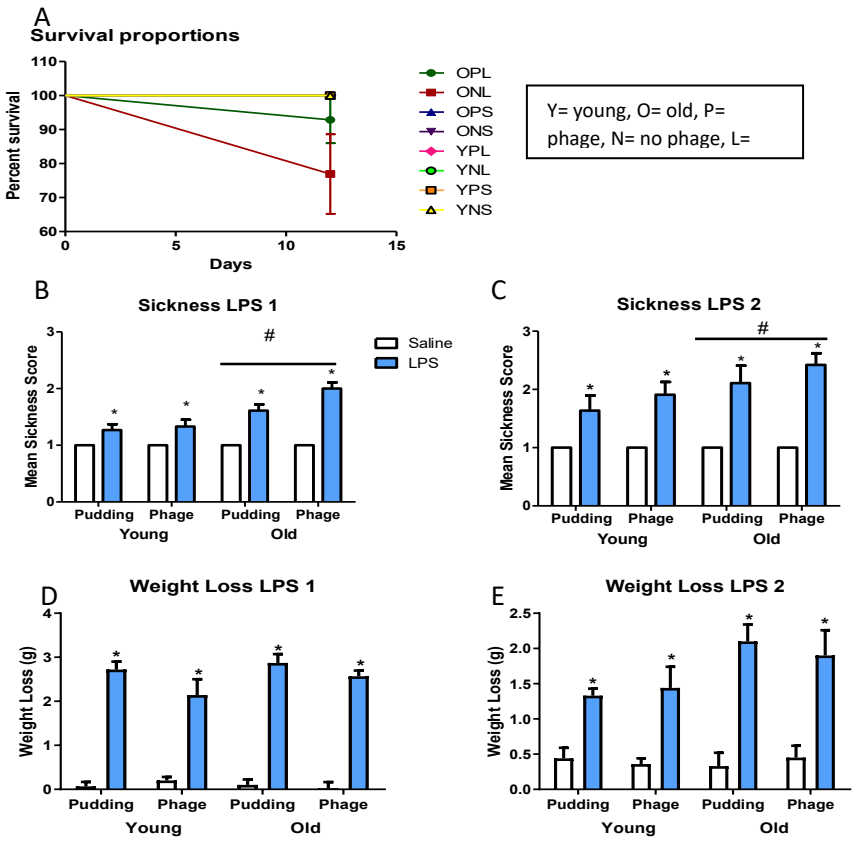


Figure 2. The LPS treatment selectively reduced survival (Panel A) and provoked marked signs of illness in the aged ("old") mice following the first (Panel B) and second (Panel C) injections.

Similarly, LPS induced the expected weight loss after the first (Panel D) and second (Panel E) administrations. The phage treatment had a modest, albeit non-significant impact on the manifestation of sickness. *p < 0.05, relative to non-LPS treated controls, # p < 0.05, relative to young mice.

Peripheral inflammation

Circulating LCN2 levels were significantly altered in an Age x Treatment manner (F (1,41) 3.36, p=0.074). Specifically, although LPS significantly increased LCN2 levels in all animals, the elevation in the older mice significantly surpassed that of the younger cohort (p < 0.05). The impact of the phage treatment on LCN2 concentration missed significance (F (1,41) 2.79, p=0.10), but there was a clear trend towards a greater rise in the phage treated mice that also received LPS.

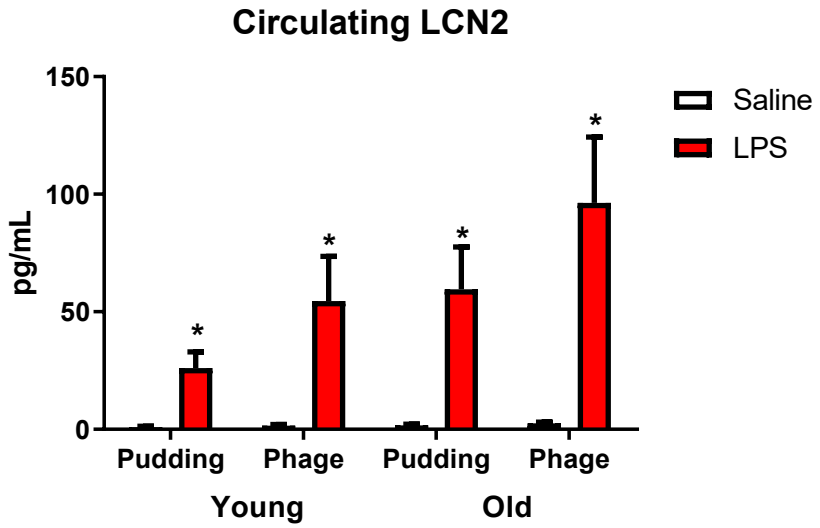


Figure 3 Circulating LCN2, as an index of inflammatory peripheral neutrophil activity. The levels of LCN2 were significantly elevated by LPS and this was especially apparent in the aged mice. Clearly, neutrophil mobilization was promoted by the endotoxin and old mice might be especially affected; yet any impact of the phage appears modest. *p < 0.05, relative to non-LPS treatment.

Immunofluorescent staining

Immunofluorescent staining was used to characterize microglia within the substantia nigra (SNc). TH was used to label adjacent dopaminergic neurons; CD68 was used as an index of microglial activation and IBA-1 was used to label microglial morphology.

There was a significant main effect for the phage treatment on microglial morphology $F(1, 32)$ 5.517, $p<0.0252$. Specifically, the phage increased microglial morphological signs of an activated state (**Figure 4**). Moreover, the LPS treatment also influenced microglia and this effect varied as a function of age $F(1, 313)$ 7.740, $p<0.009$. Indeed, although LPS increased microglial morphology ratings in all mice, this effect was curiously most dramatic in the younger mice ($P < 0.05$). Microglial that displayed the most "activated" morphology were found in the YPL (young, phage, LPS) group, wherein an ameboid shape with less ramification was evident. In contrast, the non-treated mice displayed more ramified branching, with less robust staining (e.g. YNS; young, no LPS and saline).

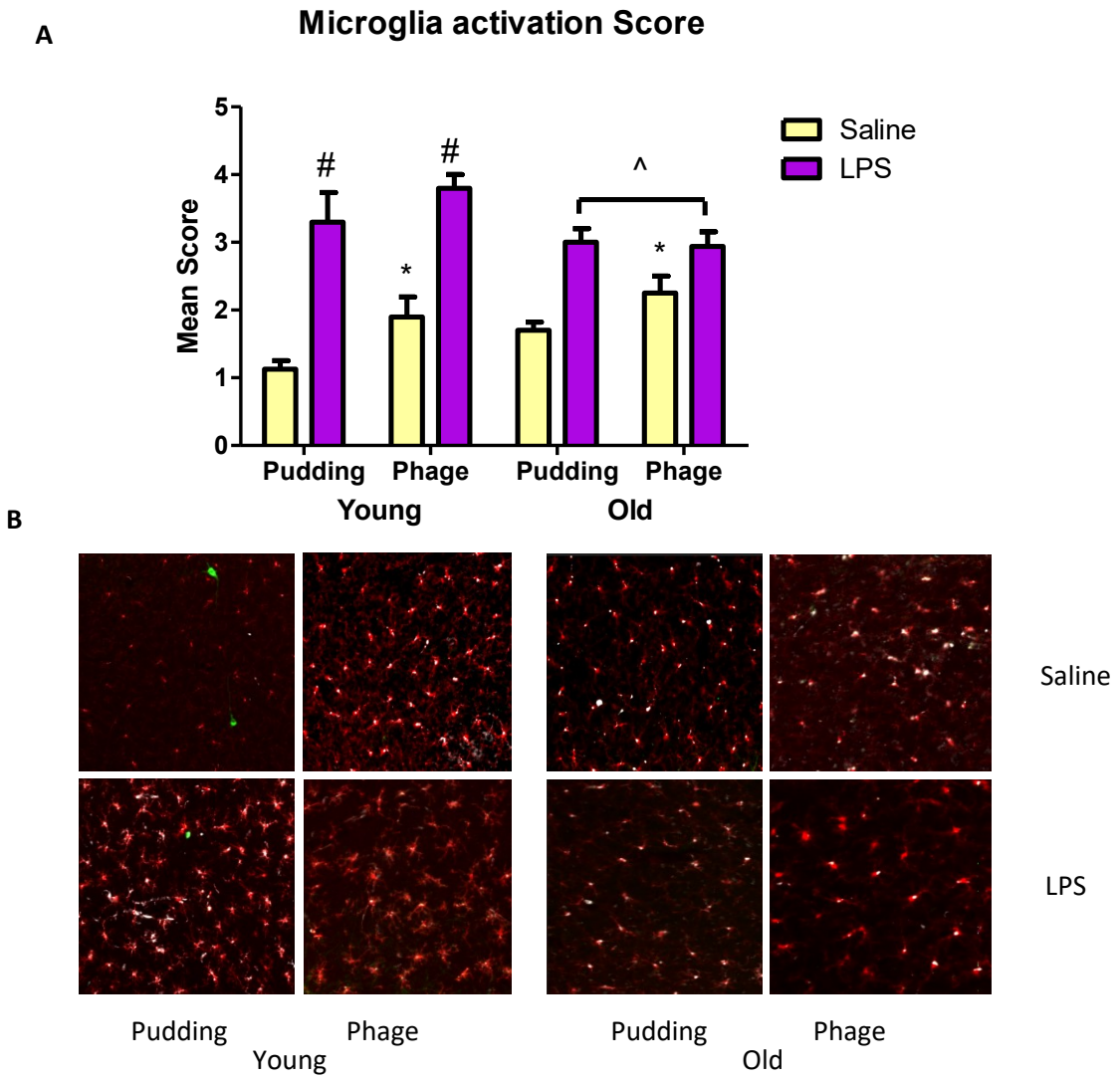


Figure 4. The LPS treatment significantly increased the activation of microglia with the most robust response seen in young mice that received phage. Old mice showed a higher level of microglial activation with control (pudding) and saline compared to young mice and although there was some activation with LPS and phage it was less pronounced, albeit still significant (Panel A). Immunofluorescent staining was used to indicate activation TH (green), CD68 (white) and IBA-1 (red) among "young" and "old" mice that received LPS or saline treatment (Panel B); *p<0.05, relative to control (pudding), # p<0.05, relative to saline treated mice, ^ p<0.05 relative to saline treated young mice.

Western blot analysis

Inflammation.

Cas1 was analyzed as an indicator of inflammation (specifically activation of the inflammasome and IL-b production) using western blot. To this end, the phage (but not age or LPS) treatment significantly altered Cas1 levels within the striatum (ST) ($F(1,40)$ 8.739, $p=0.0052$) (**Figure 5**). Indeed, the bacteriophage exposure reduced ST levels of the caspase, irrespective of age or endotoxin exposure ($p < 0.05$).

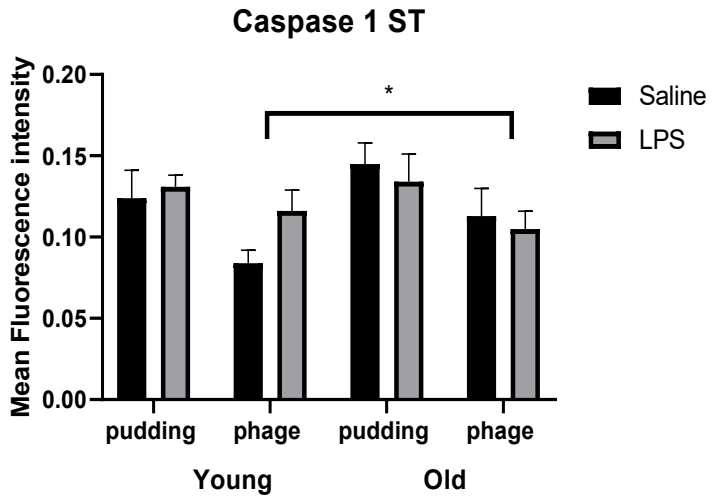


Figure 5. Increased levels of Caspase 1 indicated inflammation; however, it was significantly decreased in the striatum of mice that received phage; *p<0.05 relative to pudding (control).

Opsonization and apoptosis.

We assessed MFG-E8 since it is known to be involved in the physical aspect of neuron-microglial interactions. Accordingly, we found an Age x Treatment interaction for striatal levels of MFG-E8 ($F(1,40)$ 5.113, $p=0.0293$) (**Figure 6 A**). Intriguingly, LPS significantly reduced MFG-E8 levels but this effect was confined to the "old" mice ($p < 0.05$). Yet, it should be

underscored that the aged mice overall had an elevated basal levels of MFG-E8 ($p < 0.05$). There was no significant effect of phage.

The specific microglial marker, CX3CR1, was found to be significantly altered in striatum (ST) by the phage treatment ($F(1,40)$ 6.583 $p=.0141$) and in the SNc as a function of age ($F(1,40)$ 3.295 $p=0.0765$) (**Figure 6 B**). In both cases, (i.e. the phage and older age) resulted in significant reductions of the CX3CR1 protein ($p < 0.05$).

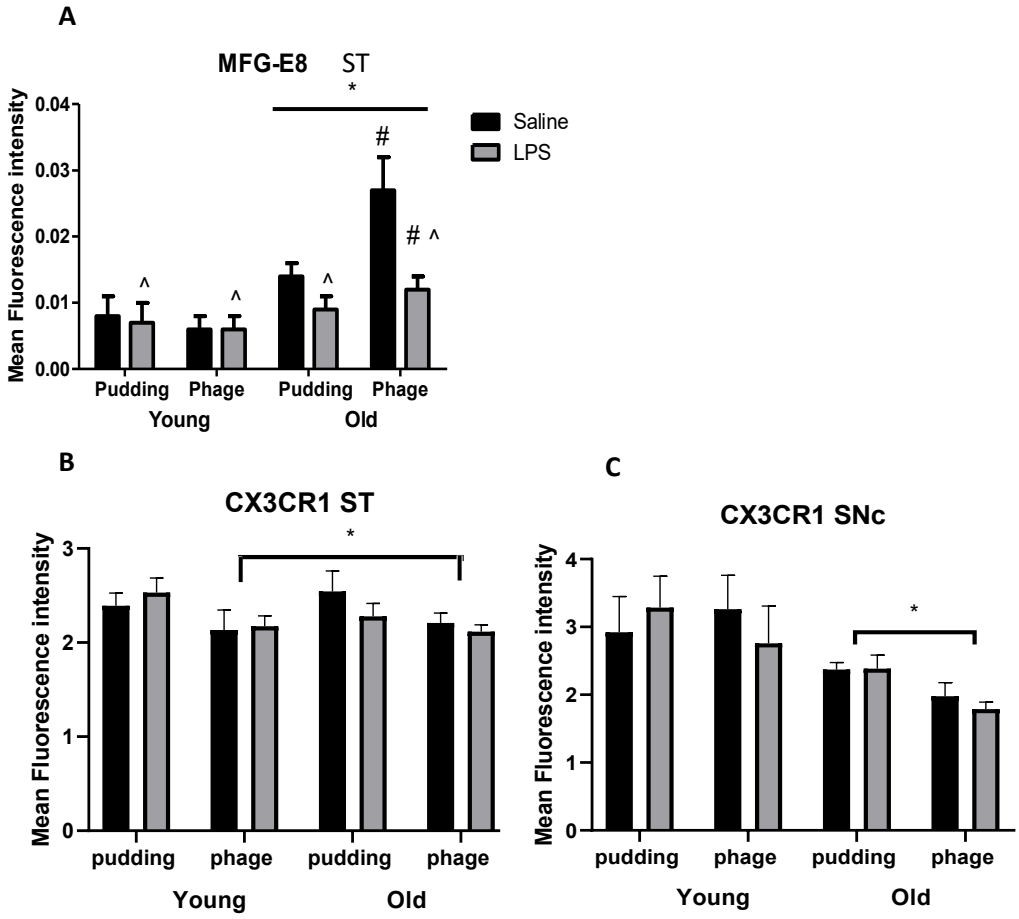


Figure 6 LPS decreased the levels of the opsonization marker MFG-E8 in the striatum of the mice. Overall the old mice had higher levels of MFG-E8 especially in mice that received phage; *p< 0.05 relative to young mice; # p< 0.05 relative to young that received pudding (control) mice; ^ p<0.05 relative to mice that received saline (Panel A). The levels of CX3CR1 where decreased both in the striatum of mice that received phage; * p< 0.0 5 relative to pudding

(control) (Panel B) and in the substantia nigra of old mice * p< 0.05 relative to young mice (Panel C).

Glial Cell Mobilization.

WAVE2 and GFAP are important proteins that regulate the cytoskeleton (and hence, mobility and morphology) in microglia and astrocytes, respectively. Levels of WAVE2 within the SNc were significantly affected by age F (1, 40) 5.575, p=0.0232) (**Figure 7 A**), with a reduction evident in the aged mice ($p < 0.05$). In contrast, GFAP levels in striatum (ST) of aged mice were actually increased, (F (1, 40) 5.64, p = 0.0224) (**Figure 7 B**).

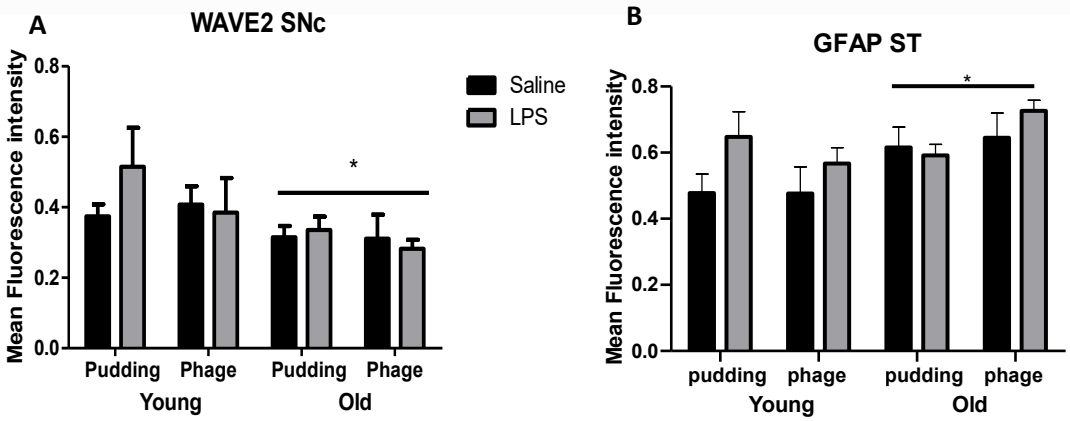


Figure 7. Old mice had decreased WAVE2 in the substantia nigra; * $p < 0.05$ related to young mice (Panel A) as well as a decrease in GFAP in the striatum; *$p < 0.05$ relative to young mice (Panel B).

Neuroprotection.

There was a significant Phage x Age interaction (F=5.645 p=0.0224). The comparisons revealed that "old" mice that received the phage had a reduction in striatal (ST) Sirt3 levels ($p < 0.05$); whereas no such effect was apparent in the younger mice (**Figure 8 A)**. In the SNc, there was a similar Phage x Age interaction. F (1, 40) 4.327, p=0.0440). Yet, this time the phage selectively decreased Sirt3 levels in the younger mice ($p < 0.05$) (**Figure 8 B**). Interestingly,

however, older mice already had basally reduced SNc Sirt3 levels, compared to the younger mice.

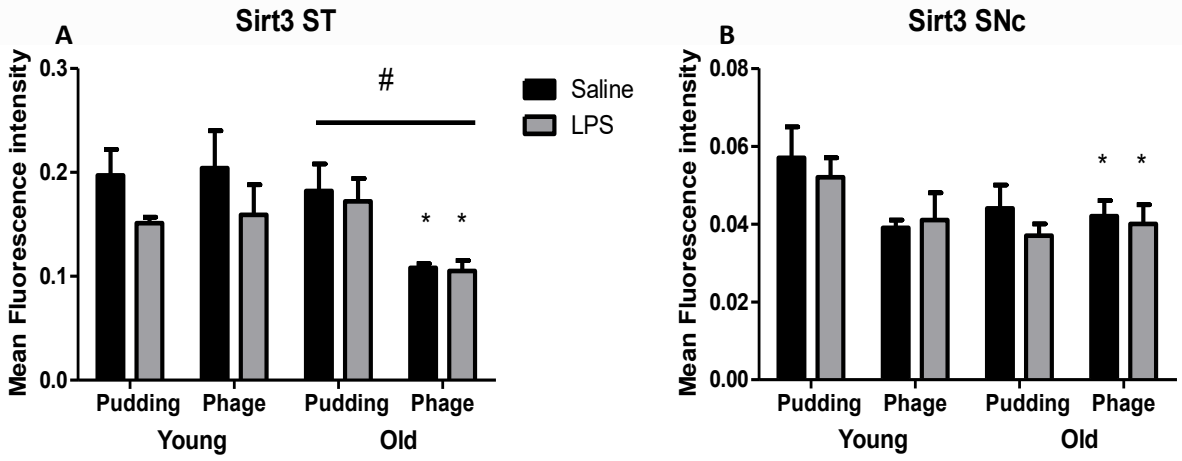
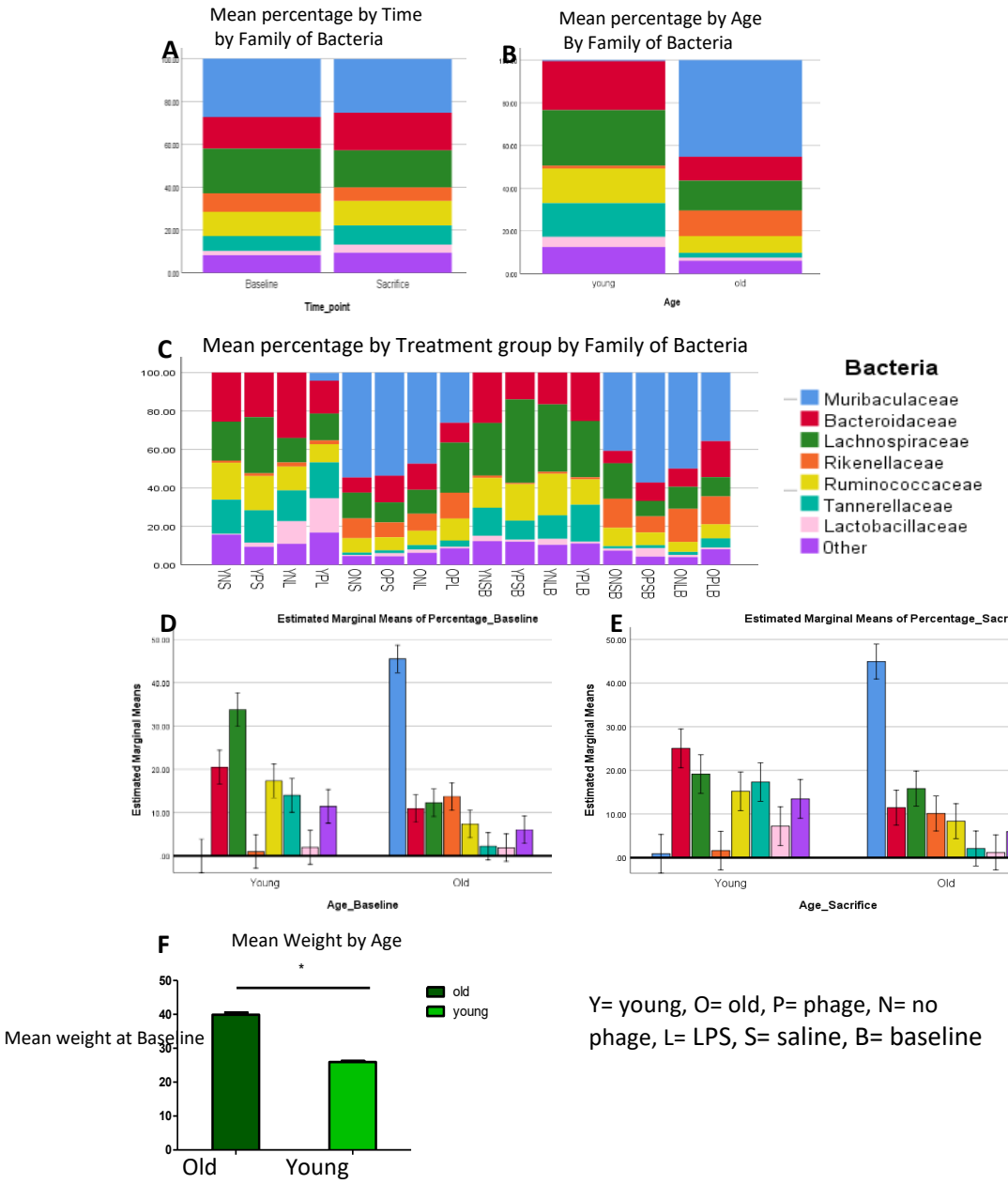


Figure 8. Old mice had less Sirt3 in the striatum (Panel A); old mice that received phage had the lowest levels of Sirt3 in both the striatum and substantia nigra (Panel B); # $p < 0.05$ relative to young mice; *$p < 0.05$ relative to young mice that received pudding (control).

Microbiota.

The LPS and phage treatments had absolutely no effect on the gut microbial species sampled and similarly, there was little variation in bacteria at family taxonomic classification simply with the passage of time over the experiment (**Figure 9 A**). However, there were marked differences in the microbiota between the old and young mice (as visualized in the stacked histogram **Figure 9 B**). The differences in microbiota between old and young mice were observed both at baseline and at the end of the experiment (i.e. time of sacrifice on Day 16) (**Figure 9 C**). The variation of bacteria at baseline (**Figure 9 D**) and at sacrifice (**Figure 9 E**) are depicted in relation to age. It can be seen that the dominant bacteria order in old mice is Muribaculaceae at both baseline and time of sacrifice; where as in young mice, Lachnospiraceae

dominates at baseline and Bacteroidaceae dominates at the time of sacrifice. In addition, old mice have more Rikenellaceae, while young have more Tannereallaeae at both time points. In addition, a physiological difference between the old and young mice is their initial weight F(1,96) 449.657 p<0.0001 (**Figure 9 F**)



Y= young, O= old, P= phage, N= no phage, L= LPS, S= saline, B= baseline

Figure 9 Microbiota variations of bacteria at family taxonomic classification, **A** The was slight variation in families of bacteria between baseline and sacrifice but a higher degree of variability young and old mice **B**; **C** further emphasis these findings. At baseline Lachnospiraceae was the dominant bacterial family in young mice; where as Muribaculaceae was dominant in old mice **D**.

At sacrifice Rikenellaceae was dominant in young mice and remained Muribaculaceae remained in old mice **E.** Old mice were significantly weighed more than the young mice **F**.

Discussion

PD has multiple contributing factors, with a popular dual theory proposing that external pathogens enter both through the nose and the gut and attack the vulnerable enteric nervous system (ENS), which eventually leads to neurodegeneration (Verreault, 2011; Horst, Laborius, & Heller, 2003; Mori, 2017). Based on previous studies, of patients with PD having abnormally high levels of dairy borne phage 936 in their gut, we examined whether this phage might influence PD-like inflammation and/or neuronal death. Indeed, inflammation is a potential causative factor of PD, with the CSF of patients with PD having elevated levels of pro-inflammatory cytokines and autoantibodies that are known to be toxic to dopaminergic (DA) neurons (Lecours et al., 2018). Previous research has focused on ways to remove it from dairy, as it is largely resistant to pasteurization and accelerates dairy spoilage. But, to our knowledge, no studies have yet examined the potential of phage 936 to cause or at least, modulate inflammation in the CNS. Our novel hypothesis is that phage 936 is a possible linking element between PD and gut dysbiosis, and that this becomes apparent with advanced age.

Increased peripheral inflammation

LPS is an endotoxin that is widely used in PD models due to its ability to induce marked microgliosis and the release of oxidative stress radicals, which have been repeatedly linked to PD (Liu & Bing, 2011). LPS has also been shown to cause long-term changes in cognition and motor functioning, that are attributable to CNS inflammatory processes (Kang et al., 2018). In the present study, we observed that LPS treatment induced marked signs of illness and weight

loss that were especially apparent in the older mice. In addition, the phage 936 also induce increased sickness rating, but appreciably more modest that what was elicited by LPS treatment.

The peripheral administration of LPS also increases circulating LCN2 levels (Kang et al., 2018; Abella et al., 2015). LCN2 is an iron trafficking protein, with antimicrobial properties, that is involved in apoptosis and the innate immune response; with elevated levels indicating neutrophil activation. (Li et al., 2018; Roach et al., 2017; Yang et al., 2002). There was also a trending increase with phage, albeit not significant. Phages in the blood are known to quickly activate and modulate the immune response (Huh et al., 2019). High levels of LCN2 is involved in the stimulation of neutrophil migration in both acute and chronic inflammation, it has also been implicated in several diseases (Shao et al., 2016; Schroll et al., 2012). Neutrophils are short lived with a span of 1-3 days and peak around day 2 of the initial infection (Jonczyk-Matysiak, 2017; McKee & Lukens, 2016). Our samples taken were within this window, 24 hrs after the final LPS injection; however, phage has been found to accelerated turnover rate of neutrophils (Huh et al., 2019) and hence, we may have missed the optimal sampling time.

The role of LCN2 in neuroinflammation is dependant on timing, individual sensitivity and level of expression (Kang et al., 2018). The peripheral administration of LPS shows an upregulation of Lcn2 mRNA, with increased LCN2 levels in the choroid plexus after an hour (Marques et al., 2007). Since many believe that PD initiates in the periphery and later moves to the CNS, the interplay between circulating immunological factors, such as LCN2, and those in the brain may be important to understanding the pathology of PD (Sawada, Oeda & Yamamoto, 2013; Mulak, & Bonaz, 2015).

Increased activation in microglia

Interestingly, the signs of peripheral inflammation that we observed did not directly translate into CNS microglia activation changes. Although LPS was still seen to activate the microglia, the young mice appeared to have a greater level of activation compared to the old mice. This may be due to the haematopoietic stem cells of old animals favoring the differentiation into myeloid over lymphoid progenitor cells (Shaw et al., 2010). Old mice having a skewed production of myeloid over lymphoid cells would cause a larger neutrophil response in the periphery but would limit microglia- lymphocytic T-cell interactions and hence, microglial activation (Schetters et al., 2018). In addition, aged animals exhibit dysregulation in neutrophils as characterized by "inflammaging", which can cause a more robust neutrophil response (Shaw et al., 2010). Age related immunosenescence of microglia, reduction in TLR receptors and dysregulation of signalling pathways, may also account for the less robust activation (Shaw et al., 2010; Shen et al., 2019). In contrast, the increased activation in the young mice may be due a persistent response by the microglia; they can become activated like hematopoietic macrophages yet lack negative regulator pro- IL-1B (Burm et al., 2015; Henry, Huang, Wynne & Godbout, 2008).

Dysregulation of Caspase 1

We found that phage 936 decreased levels of Cas1 in the ST, signifying potential dysregulation of proinflammatory and antimicrobial responses (Bauernfeind & Hornung, 2013). Indeed, Cas1 is an evolutionary conserved enzyme and phage 936 may have evolved adaptations to suppress the immune response in order to promote its survival. When Sweere et al. (2019)

infected a wound with bacteria containing phage, they found an inappropriate antiviral response, decreased bacteria clearance and the presence of phage RNA.

Cas1 is recruited when inflammasomes identify microbial associated molecular patterns (Skeldon & Saleh, 2011; Voet, Srinivasan, Lamkanfi & Van Loo, 2019). The nucleotide binding oligomerization domain (NOD)- like receptor (NLR) acts as a scaffold protein for the inflammasome and recognizes viral components (Lamkanfi et al., 2008). In addition to pathogens, NLRs also sense large protein aggregates and neighboring cell death and have been implicated in PD (Burm et al., 2015). Specifically, microglia activation through NLRs have been implicated in neurodegenerative diseases; NOD2 specifically, caused dopamine degeneration in the 6 hydroxydopamine model of PD (Burm et al., 2015; Cheng et al., 2018). Interestingly, as in PD, it is alterations in NOD2 function that can also lead to irritable bowel disease (Saleh & Trinchieri, 2011).

Once activated, Cas1 proceeds to cleave IL-1B and IL-18 and releases alarmins to facilitate cell death through pyroptosis (Skeldon & Saleh, 2011). This mechanism impedes pathogen replication and dissemination; however, evolutionary adaptions have allowed pathogens to target and evade Cas1; including, influenza NS1, poxvirus PYD, tuberculosis, cholera and typhoid (Skeldon &Saleh, 2011; Saha et al., 2019). Some pathogens can down-regulate their expression of immune markers, such as PAMPs, in an effort to evade Cas1; similarly, some have argued that phages may also act like other pathogens or viruses by having the ability to change capsid proteins and other elements that foster their survival (Ernest & Guina, 2005; Finlay & McFadden, 2006).

Phage 936 appears to also have the ability to target and evade Cas1, potentially to increase its rate of survival. Although activated Cas1 is pro-inflammatory, its deficit does not necessary inhibit the Il-1B inflammatory pathway as other protease can produce active IL-1B in

its absence (Guma et al., 2009). IL-1a, the product of a different gene, binds to the same receptor as Il-1B to promote inflammation (Li et al., 1995). There are several redundant mechanisms and cytokines can be generated without Cas1 or TLR signalling (Lara-Tejero et al., 2006; Mayer-Barber et al., 2010). These redundant pathways may account for the observed decrease of Cas1 in the ST of phage mice, while still visually showing signs of inflammation.

Dysregulation in opsonization and apoptosis

Inflammation can cause apoptosis and an increase in opsonization (coating of an antigen with antibody, thereby labelling it for destruction). We examined MFG-E8 as a representative marker for opsonization. MFG-E8 is an opsonin glycoprotein that has a role in maintaining homeostasis in several areas including the gut, blood and brain (Oshima et al., 2014; Kaminaska, Enguita & Stepien, 2018). MFG-E8 is key to the removal of apoptotic cells, debris (efferocytosis), microvesicles, prions and a variety of unwanted molecules (Fricker et al., 2012; Kaminaska et al., 2018; Oshima et al., 2014; Kraniche et al., 2010; Kinugawa et al., 2013; Yi, 2016). It bridges the phosphatidylserine that becomes exposed in apoptotic cells, with the integrins of phagocytic cells, such as microglia (Fricker et al., 2012; Miksa et al., 2008). In addition to its clean-up role, it also promotes cell migration and proliferation, effects neutrophil infiltration and maintains gut mucosal integrity under physiological stress (Cheyuo, Aziz & Wang, 2019; Bu, Zuo & Wang, 2007). We presently found a decrease in MFG-E8 in LPS treated mice, but an increase in older mice, particularly those treated with the phage.

Our findings, that LPS decreased levels of MFG-E8 in young mice is consistent with other studies that found that LPS decreases MFG-E8 and takes an anti-inflammatory role in the LPS-TLR4 response (Yi, 2016). Indeed, Li et al. (2019) found that MFG-E8 regulates microglia

and can have antioxidant effects through the Keap-1/Nrf-2/HO-1 pathways during neuroinflammation induced by LPS. Similarly, Aziz et al. (2011) found that animals pretreated with recombinant murine MFG-E8 followed by LPS had a significantly down-regulation of LPS -induced NF-kB p65 and TNF-α. Miksa et al. (2008) found that in a co-culture of apoptotic cells and macrophages, MFG-E8 suppressed both intracellular and extracellular signalling molecules as well as the activity of p65 in the NFkB pathway. However, Fricker et al. (2012) found that MFG-E8 was required for the phagocytosis of live neurons when inflammation was induced with LPS. It is proposed that after an LPS challenge, apoptotic cells increase TGFβ1 expression in microglia, resulting in a shift in favor of the alternative over the classic pathway. In this case, MFG-E8 no longer acts directly on the microglia (Spittau et al., 2014). A pathological decreased MFG-E8 levels causes delayed clearance of apoptotic cells, which creates autoantibodies due to the build up of cellular debris, toxic proteins accumulation and increased inflammation (Yi, 2016; Sokolowski & Mandell, 2011; Mukundan et al., 2009).

MFG-E8 naturally increases with age, which is consistent with our age-dependent rise of the factor within the brain (Xu et al., 2017). In fact, due to its role as an inflammatory mediator, is considered a hallmark for age associated cardiovascular remodeling and the amyloidization of the extracellular matrix (Wang et al., 2013). Despite increased levels potentially being helpful by decreasing inflammation through the phagocytizing of inflammatory cytokines, it can also be destructive, as is evidenced by its ability to phagocytize live neurons (Liu et al., 2013; Neniskyte & Brown, 2013). The role of MFG-E8 in PD remains a target of investigation; for example, current PD research is examining the role of MFG-E8 in the differentiation of stem cells to dopaminergic cells (Zhou et al., 2018; Nakashima, Miyagi-Shiohira, Noguchi & Omasa, 2018).

While MFG-E8 is an opsin that encourages apoptosis, CX3CR1 participates in inflammatory signal and in certain cases, might contribute to apoptosis (Limatola & Ransohoff, 2014; Wang et al., 2018). It is a receptor for the chemokine, fractalkine abbreviated CX3CL, and is exclusively expressed in microglia and peripheral immune cells (Shi et al., 2019). Typical chemokines are small secreted proteins that bind to extracellular matrices to form gradients, but CX3CL1 is a transmembrane protein that presents its chemokine domain to permit tight adhesions to CX3CR1 leukocytes (Landsman et al., 2009). CX3R1 are found in natural killer cells, monocytes, T- cells, microglia, neurons and astrocytes (Brand, Sakaguchi, Gu, Colgan & Reinecker, 2002). When fractalkine is cleaved by metalloproteases it is released to recruit effector T- helper cells and regulate intraepithelial lymphocytes (Landsman et al., 2009).

CX3CL1-CX3CR1 is a key communication system between neurons and microglia, its signalling regulates many functions; including, synaptic plasticity and cognitive function (Mecca, Giambanco, Donata & Arcuri, 2018). Consistent with our findings, dysregulation of this system may occur with aging; since aged mice have decreased fractalkine production and microglia deficient in CXCR1 have impaired phagocytosis and migration (Wynne, Henry, Huang, Cleland & Godbout, 2010; Castro-Sanchez, Garcia-Yague, Kugler & Lastes-Becker, 2019). Also consistent with our findings, Wynne et al. (2010) found that aged mice given injections of LPS had decreased CX3CR1 and prolonged sickness behaviors compared to young adult mice (**Figure 2** & **Figure 6 C**). The reduction of CX3CL1 in the brain of aged mice may contribute to the dysregulation of microglia and thus increase the risk of neurodegeneration (Wynne et al., 2010).

We also found significant decreases of CX3CR1 in the ST of mice that received phage 936, this could conceivably indicate a loss of normal protective functions. It is speculated that phage

936 may be perceived as a virus, triggering an immune response. Indeed, CX3CR1 is associated with viral infections; including, HIV, viral encephalitis, and respiratory syncytial virus. (Foussat et al., 2001; Kaufer et al., 2018; Johnson et al., 2015).

Decreased cell mobilization with glial activation

GFAP is a biomarker for astrocyte activation and WAVE2 is a cytoskeleton protein that is necessary for movement in microglial cells. Together they are indicative of glial activation, mobilization or senescence. In particular, WAVE2 is fundamental to cytoskeleton reorganization, actin polymerization regulation, tissue repair, immune response, embryonic development, migration and cell motility; it is particularly important in the formation of lamellipodia (Joseph et al., 2017; Oikawa et al., 2004).

Based on our findings that WAVE2 decreases in the SNc of old mice, microglia may also lose WAVE2 with age and hence, lose their ability to an appreciated respond to relevant challenges. Indeed, although there is limited information about WAVE2 in relation to aging glial cells, its reduction in relation to age has been found in oocytes and T-cells (Sun et al., 2012; Gonzalo & Miller, 2011). WAVE2 utilizes actin to create directional movement in the lamellipodia (Jia et al., 2014). Aging T-cells lose cytoskeleton structure and membrane fluidity as filamentous actin polymerizes; cell mobility depends on the ability to re-arrange actin filaments (Gonzalo & Miller, 2011; Jia et al., 2014; Pauker et al., 2014). Since actin is the main component of the cytoskeleton and the most abundant protein in the eukaryotic cell, its polymerization causes dystrophy in aged microglia (Cao, Yao & Zhang, 2015). The morphological changes in microglia that are associated with age may be due to WAVE2 deficits causing defective migration and lamellipodia formation (Lecours et al., 2018; Joseph et al., 2017).

GFAP is a biomarker of astrocyte and enteric glial cell (EGCs) activation; it allows for the formation of processes to communicate with neurons and the strengthening of the gut-brain axis. A single astrocyte modulates and supports an estimated 2 million synaptic connections in humans (Brachmachari, Fung & Pahan, 2006.; Coelho-Aguiar et al., 2015; Weinstein, Shelanski & Liem, 1991; Oberheim, Wang, Goldman, Nedergaard, 2006). The neuroglial junctions, calcium signals and neurotransmitter receptors on EGCs, allows for communication with the ENS (Sharkey, 2015; Ochoa-Cortes et al., 2016). Dysfunction of each of these has been implicated as a possible source for gut mobility issues and possibly even neurodegeneration (Coelho-Aguiar et al., 2015; Ochoa-Cortes et al., 2016; Sequella, Capuana, Sarnelli, & Esposito, 2019). Furthermore, EGCs have been proposed to serve as a reservoir for prions and aberrant alpha-synuclein; with several studies showing that these molecules are readily able to travel from the gut to the brain and vice versa (Albanese et al., 2008; Sharkey, 2015).

Our findings show that GFAP is increased in the ST of "old" mice is important since activated astrocytes have been implicated in PD (Setkowicz, Kosonowska & Setkowicz, 2017; Liddelow et al. 2017). Interestingly, GFAP is over- expressed, along with being hypo-phosphorylated at serine 13 in the EGCs of patients with PD, compared to healthy individuals (Claireembault et al.,2014). Increased GFAP with age is consistent with previous studies, with findings that astrocyte activation increases cellular demands, potentially at the expense of neurons, as evidenced by age associated decreases in neuronal mitochondrial metabolism but increased glial mitochondrial metabolism (Boumezbeur et al., 2010; Cotto, Natarajaseenivasan & Langford, 2019). Increased GFAP also causes an upregulation in antigen presenting pathways, complement activation, cytokine response and neurodegeneration; particularly in the striatum (Cotto et al., 2019).

Decreases neuroprotection

Old mice have been reported to display decreases in Sirt3; rendering them vulnerable to toxic challenges that impact the mitochondria (McDonnell, Peterson, Bomze & Hirschey, 2015). Sirt3 is a mitochondrial de-acetylate that influences almost every pathway in the mitochondria and plays a primary role in stress resistance, metabolism, neurodegenerative disease and aging (McDonnell et al., 2015; Ansari et al., 2017; Anamika, Khanna, Acharjee, Acharjee, & Trigun, 2017; Budaveva, Rowland & Cristea, 2016). Mitochondrial dysfunction is linked to aging due to being both a source and target of ROS (Kincaid & Bossy-Wetzel, 2013), Sirt3 can eliminate ROS and prevent apoptosis; hence, it is generally thought to be neuroprotective (McDonnell et al., 2015).

McDonnell et al. (2015) found that Sirt3 KO mice displayed an acceleration in age associated diseases; such as, metabolic syndrome, cardiovascular disease, cancer and neurodegeneration. Studies are currently underway to determine if elevating levels of cellular Sirt3 would improve mitochondrial health and reduce cell death and thus protect against PD (M.J. Fox, 2019). Consequently, when microglia activation reduces Sirt3 in dopaminergic neuronal cells, ROS can irreversibly damage lipids, proteins and DNA in these neurons (Ansari et al., 2017; Jiang et al., 2019; Kincaid & Bossy-Wetzel, 2013).

The lowest levels of Sirt3 were evident in old mice that also received the phage treatment. Viruses have been known to appropriate Sirt regulatory pathways through the manipulation of host epigenetic and transcriptional changes (Budaveva et al., 2016). For example, Influenza A evolved to encode NS1 to mimic histone H3 and suppress antiviral genes; thus, Sirt inhibition increased Influenza A titers (Budaveva et al., 2016). Although, to our

knowledge, there is no literature on how phages, would affect Sirt3, our findings suggest that phages may induce similar effects as that of a viral infection.

Dysbiosis and bacteriophages

Many of the biomarkers found in the brain are also found in the gut; thus, reinforcing the possibility of the gut brain axis in the pathogenesis of PD. For example; intestinal epithelial cells express many of the same inflammasome components and cytokines that microglia express (Sellin, Maslowski, Maloy, & Hardt, 2015). CX3CR1/ CX3CL1 is also an autocrine regulator of intestinal epithelial immune function; including, regulating immune cell migration and their interactions with intestinal epithelial cells and the mucosa (Brand et al., 2002). It has also been implicated in gut inflammation, with high levels of fractalkine in gut lymphoid and inflamed colonic tissue (Foussat et al., 2001; Kuboi et al., 2019).

Inflammation can cause gut dysbiosis with pathophysiological consequences, with mounting evidence that peripheral inflammation is often initiated in the gut and then precedes to the CNS. Along these lines, irritable bowel disease (IBD) is positively correlated with parkinsonian disorders (Villumsen, Aznar, Pakkenberg, Jess & Brudek, 2017). Due to the immunomodulating properties of phages, some studies have indicated that phages may contribute to the development of IBD while others seek it as a potential treatment (Łusiak-Szelachowska, Weber-Dąbrowska, Jończyk-Matysiak, Wojciechowska & Górski, 2017; Gorski et al., 2018). This discrepancy may be due to the characteristics of specific phages or their administration paradigm. Since phages have been shown to modulate the B and T cells, as well as innate immune responses it has been suggested that they could be used to treat autoimmune and immunosuppressant diseases (Łusiak-Szelachowska et al., 2017).

Phages are invasive and innately penetrate eukaryotic cells throughout the body; including, the epithelial layers of the gut, the BBB and areas once considered sterile (Nguyen et al., 2017). In 1943 Dubos et al. found that it only took one hour for phages to accumulated in the brain after mice received an IP injection of phage (Huh et al., 2019). Phages can also infiltrate immune cells; phages can attach to the membrane of leukocytes and promote lytic activity within the cell (Jonczyk-Matysiak et al., 2017). Despite phages being discovered in 1915 little is known about the impact of phages on animals (Clokie et al., 2011). Phages are biodiverse and only a limited number of existing phage types, mostly M13, have been studied in lab or used for engineering (Pires et al., 2016). There are compelling uses for genetically engineered phages; including, treatment of antibiotic resistant diseases and for diagnostics such as the detection of aberrant alpha-synuclein (Pires, Cleto, Sillankorva, Azeredo & Lu, 2016; Woods et al., 2007). Moreover, Sergeev et al. (2019) found that phages had a significant effect on the expression of alpha-synuclein in lymphocytes and intestinal neurons, raising the possibility that targeted bacteriophage treatments could ostensibly impact the spread of Lewy bodies. Yet, the complexities of the inter-connected CNS-microbiome network makes it likely that any such treatments might have cascading and possibly unintended effects.

Fecal DNA

Commensal microorganism within the gut do not normally cause an inflammatory response, but rather the innate immune system "cross-talks" with the microbiota to regulate intestinal homeostasis through mucosal regeneration and epithelial turnover and autophagy (Saleh & Trinchieri, 2011). Since we did not detect appreciable levels of *Lactococcus lactis (L. lactis),* the host bacteria for phage 936, we can determine whether the phage directly effected the immune

response; rather than, secondarily through subsidiary host bacteria. *L. lactis* is not typically found within the mouse gut, despite being prevalent in the human gut (Ley et al., 2005; Nguyen, Vieira-Silva, Liston & Raes, 2015). To compensate for this, our subsequent studies will administer supplemental *L. lactis* prior to further study. The addition of *L. lactis* would allow exponential increases of phage in the mice and the effects and consequences on the microbiome could be further examined.

L. Lactis shares the taxonomic classification at the family level with the gut microbiota present; namely, Lactobacillaceae which subsequently diverges into various genus. Further analysis was performed at the Family level for consistency. Findings showed variation in the bacteria related to age at both baseline and sacrifice (**Figure 9A, 9B & 9C**). Lachnospiraceae substantially decreased in the young mice from baseline to sacrifice in all treatment groups. Interestingly, Lachnospiraceae levels are inversely related to the risk of PD (Keshavarzian et al., 2015). Many butyrate producing bacteria belong to this family and there is evidence that decreased levels contribute to leaky gut and gut inflammation (Keshavarzian et al., 2015). The predominant bacteria in the old mice was Muribaculaceae (**Figure 8 D & 8E**). There is evidence that some members of this family can initiate an innate immune response, thus impacting gut function and health (Ormerod et al., 2016). Although there are variations within the Muribaculaceae (S24-7) family, its abundance is largely associated with high fat diets (Ormerod et al., 2016). The old mice in the study were relatively sedentary and despite consuming regular chow would generally be considered obese even at baseline (**Figure 9F**) . Though obesity is not the focus of this study it is a relevant feature of aging as sarcopenia is a natural part of the aging process (Paddon-Jones et al., 2008).

Bacteroidaceae was observed in all mice, with the highest levels in young mice at baseline; it is a common gut bacterium, but it does not exist in isolation, but rather interacts with other bacteria. Bacteroidaceae has inhibitory interaction with Lactobacillaea as well as Ruminococcace but positive interactions with Rikenellaceae as well as Tannereallaeae (Lessesen Consulting, 2019). These interactions and composition of the gut microbiota and virome create different enterotypes with unique characteristics (Nguyen et al., 2015; Metzger, Krug & Eisenacher, 2018). Potentially due to this interplay, Rikenellacea steadily increases throughout the lifespan of mice (Bauerl, Collado, Cuevas, Vina & Martinez, 2018). Rikenellacea is considered healthy at low levels, but increases can cause dysbiosis (Thomas et al., 2011). Dysbiosis can also occur with the decline in bacterial diversity; in this regard we found that the old mice had a less diverse microbiome compared to the young mice (Kriss et al., 2018). Ultimately, both age associated dybiosis can increase intestinal permeability and systemic inflammation both of which are relative to the development of PD (Thevaranjan et al., 2017).

Conclusion

We have provided evidence that phage alone does cause measurable changes in inflammatory biomarkers, both peripherally and centrally. We also determined that phage caused behavioral changes, as evidenced by sickness scores and weight loss. It could therefore be concluded that our hypothesis was reasonable; both LPS and age did exacerbate the immunological changes produced by phage, thus producing detectable pathology in mice. However; unexpectantly, phage was able to elicit some immunological and behavioral changes on its own without the additional stressors. Furthermore, even without the host bacteria being

present, phages could directly influence the immune response; this may be due to the evolutionary acquisition of mechanisms able to circumvent pathways that would affect their proliferation or cause their demise.

In addition, results relating to the old mice were consistent with “inflammaging”, with increased inflammatory biomarkers and dysbiosis. PD is considered a geriatric disease that causes neurodegeneration and altered mobility; mounting evidence implicates the gut brain axis as being of paramount importance in this multifaceted disease. Phage 936 resides in the gut where it proliferates and destroys its probiotic host L. lactis. We have demonstrated that despite residing in the gut it can cause dysregulatory effects within the brain. We present for the first time, that phage 936, in aged mice may have complex effects that vary with the presence of inflammation (e.g. induced by LPS).

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