The role of the LRRK2 G2019S mutation in neuroinflammatory processes and its relevance for Parkinson's disease

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Abstract

Parkinson's disease (PD) has been linked to a variety of environmental and immunological challenges involving both oxidative and inflammatory processes, however, genetics also clearly plays a role in vulnerability to such insults. Indeed, the Gly-2019-Ser (G2019S) leucine rich repeat kinase (LRRK2) mutation is the most common mutation enriched compared to controls in both familial and sporadic Parkinson's patients. Our current study focuses on the interplay between the G2019S genetic mutation and exposure to the bacterial endotoxin, lipopolysaccharide (LPS). In the current study mice were centrally infused with either vehicle or one of two doses of LPS (2 or 10ug) in order to determine whether an inflammatory based insult would produce augmented pathology in G2019S mutant mice. Infusion of LPS into the substantia nigra pars compacta (SNc; the region containing dopamine neurons which degenerate in PD) produced a (non-statistically significant) trend towards a dose-dependent reduction in SNc dopamine neurons in both G2019S mutants and their wild type littermates. Although few motor deficits were apparent (likely a reflection of the modest changes in dopamine neurons), the G2019S mutants did display a basal difference in gait. As would be expected, LPS infusion also induced marked sickness behaviour along with weight loss; however, G2019S mutant mice showed a slower recovery of weight days following the infusion. Interestingly, the lower of the two LPS doses (2 μg) increased microglial activation and this effect was only apparent in the G2019S mutant mice. Overall, the present results do not support a major impact of G2019S LRRK2 mutation in increasing vulnerability to the neurodegenerative or motor effects of the immunogenic insult, LPS. That said, LRRK2 G2019S mice might have increased vulnerability to non-motor co-morbid or pro-inflammatory effects of immune challenges along with displaying subtle basal motor deficits.

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List of Abbreviations

PD	Parkinson's Disease
SNc	Substantia Nigra
DA	Dopamine
DAT	Dopamine Transporter
TH	Tyrosine Hydroxylase
CD68	Centre of Differentiation 68
BBB	Blood Brain Barrier
LPS	Lipopolysaccaride
ROS	Reactive Oxygen Species
iNOS	Inducible Nitric Oxide Synthase
NF-κB	Nuclear Factor κB
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
DAB	Diaminobenzidine
MyD88	Myeloid Differentiation Factor-88
MHC	Major Histocompatibility Complex
IL	Interleukin
IFN	Interferon

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1. Introduction:

1.1 Parkinson's Disease: Background

Parkinson's disease (PD) is a progressive neurodegenerative disorder with a lifetime prevalence of approximately 1%, the second most prevalent neurodegenerative disorder after Alzheimer's disease (de Lau & Breteler, 2006). PD is behaviourally well characterized and is known to be the result of specific neuronal loss (Anderson, 2004; C W Olanow & Tatton, 1999; Schapira & Jenner, 2011; Tatton et al., 2003). Clinically PD has historically been acknowledged as a motor disorder characterized by four principle symptoms: bradykinesia, tremors, rigidity and postural instability (Anderson, 2004). However, there are also many other non-motor symptoms including olfactory dysfunction and gastrointestinal disturbances (Dujardin et al., 2014; Mollenhauer et al., 2013). Psychiatric illnesses; including depression, have also recently been found to be co-morbid with PD (Marsh, 2013; Qureshi, Amspoker, Calleo, Kunik, & Marsh, 2012). PD is a progressive disease which evolves distinctly within each patient typically with non-motor symptoms appearing early, prior to diagnoses, and cognitive symptoms emerging later either just before or during motor diagnosis (Chaudhuri, Healy, & Schapira, 2006).

The cardinal motor features of PD result from the loss of dopamine (DA)-producing neurons in the substantia nigra pars compacta (SNc), a region of the brain associated with and projecting prominently to the caudate putamen which itself is a region within the basal ganglia otherwise known as the striatum in rodents (Anderson, 2004). Briefly, the basal ganglia is a region of the midbrain principally associated with voluntary motor movements, more specifically, it plays a role in 'action selection', the process of determining which neural pathways should be used to produce the desired movement (Leisman, Braun-Benjamin, & Melillo, 2014; Obeso,

Rodriguez-Oroz, Stamelou, Bhatia, & Burn, 2014). Thus, basal ganglia dysfunction results in many of the primary motor symptoms thought to characterize PD including bradykinesia and tremors (Hughes, Altena, Barker, & Rowe, 2013).

The majority of cases of PD are said to be idiopathic, wherein the exact etiological agents or processes are unknown, however, a limited number of cases have been shown to stem from genetic alterations and an even smaller number have been directly linked to specific environmental exposures (C W Olanow & Tatton, 1999; Schapira & Jenner, 2011; Tatton et al., 2003). Current outlook tends to favour a multi-hit hypothesis, wherein multiple environmental insults superimposed upon some genetic vulnerability lead to the development of disease (Alcalay et al., 2014; Barrett, Hac, Yan, Harrison, & Wooten, 2015; Hou, Tian, Li, & Yuan, 2014; Litteljohn, Mangano, et al., 2010; Mangano & Hayley, 2009). Most evidence has implicated pesticides, heavy metals and infectious agents as being important etiological vectors (Landrigan et al., 2005; Litteljohn, Mangano, et al., 2010). Importantly, these environmental hits occur over extended periods of time and may cause cumulative damage that eventually manifests in clinical pathology after some threshold of neuronal loss/damage has occurred (Landrigan et al., 2005). Indeed, PD is generally not diagnosed until 50-70% of DA neurons have been lost and upwards of 80% of normal DA release is disrupted (Bernheimer, Birkmayer, Hornykiewicz, Jellinger, & Seitelberger, 1973; Riederer & Wuketich, 1976; Sulzer, 2007).

Besides external environmental insults, recent research has demonstrated that alterations in endogenous factors, most notably the gut microflora, occurs in PD patients (Lin, Lin, Liu, Chang, & Wu, 2014; Scheperjans et al., 2014). Indeed, PD patients have elevated levels of *Enterobacteriaceae* along with alterations in the ratio of specific gut microflora species

(Scheperjans et al., 2014). Importantly, many of these gut species have been linked to other disorders that have an inflammatory component, including irritable bowel syndrome, Crohn's disease and allergies (Noval Rivas et al., 2013; Scheperjans et al., 2014). Although the nature of the link between PD and gut microflora has not yet been established, there are several mechanistic routes that should be considered. Among these, most available evidence suggested that the critical role of the microflora in the initial development of immunity early in life and later refinement in adulthood is one primary mechanism that could have tremendous ramifications for PD. Indeed, substantial evidence indicates that the microflora species directly influence immune system maturation and development of immunological tolerance (Dupaul-Chicoine, Dagenais, & Saleh, 2013; Ivanov et al., 2009; Kamada et al., 2012). Such processes are intimately associated with the release of soluble cytokines, in fact, gut macrophages play a vital role in maintaining immune homeostasis by producing anti-inflammatory cytokines, such as IL-10 (Denning, Wang, Patel, Williams, & Pulendran, 2007; Murai et al., 2009; Nenci et al., 2007). Upcoming sections of this thesis will delve further into how immune factors might play a role in PD.

Despite the wide prevalence and rapidly aging populations in Western nations, treatment strategies for PD are currently limited to drug therapies which temporarily manage select symptoms, lifestyle changes or in the most severe cases invasive neurosurgical alterations (Goodwin, Richards, Taylor, Taylor, & Campbell, 2008; PD MED Collaborative Group, 2014; Sung et al., 2014). Broadly, the drug therapies utilized in the treatment of PD seek to replace the DA lost in the basal ganglia. This is accomplished through the administration of DA precursors, such as levodopa, dopamine agonists, such as ropinirole, enzymatic MAO-B inhibitors and anti-

cholinergics (Baba et al., 2012; Chung et al., 2015; Park, Park, Yang, & Oh, 2013). These drugs have a large number of severe side effects including confusion, hallucinations, nausea, low blood pressure and diarrhea. In addition, these therapies are only effective for a limited time and there have been several controversial studies suggesting DA replacement drugs are converted into reactive oxygen species (ROS) which can contribute further to neurotoxicity to DA neurons further propagating disease progression (Olanow et al. 2004; Blunt et al. 1993; Jenner and Olanow 1996).

Neurosurgical alterations are a form of treatment reserved for more severe cases, they include deep brain stimulation (DBS) and targeted ultrasound. DBS uses a surgically implanted pulse-generator which sends electrical stimuli to dysregulated brain regions selected based on magnetic resonance imaging (MRI) and computer tomography (CT) scans (Bötzel et al., 2006; Rodriguez-Oroz et al., 2005). The electrical stimulation is aimed at normalizing neural firing within basal ganglia regions including the thalamus, sub-thalamic nucleus and global pallidus (Universitaria, 2001). While the procedure itself is relatively safe, DBS has been reported to increase the incidence and severity of cognitive symptoms ranging from dementia and mild cognitive impairment to affective disorders (Abboud et al., 2015; Massano & Garrett, 2012; Smeding, Speelman, Huizenga, Schuurman, & Schmand, 2011). These limitations, coupled with the cost of surgery and post-operative follow-up, greatly restrict the viability of DBS as an effective treatment option.

Focused ultrasound is the second surgical option for PD, wherein the procedure involves selective lesioning of dysregulated regions in the basal ganglia (Dobrakowski, Machowska-Majchrzak, Labuz-Roszak, Majchrzak, & Kluczewska, 2014). Ultrasound was first used as a PD

treatment early in the 20th century before being phased out in favour of medication due to ineffectual results. However, recent advances in robotics and imaging have allowed focused ultrasound to be used to selectively destroy cancerous cells in several locations in the body with great precision and human trials have been undertaken to once again apply the technique to PD (Bauer et al., 2014; J. Der Lee et al., 2013). Typically, the dopaminergic projections from the SNc inhibit regions of the basal ganglia, and consequently the degeneration of these projections results in non-specific motor activation giving rise to the characteristic tremors observed in PD (Ridderinkhof & Brass, 2015; Tremblay, Worbe, Thobois, Sgambato-Faure, & Féger, 2015). Ablating the disinhibited or "over-active" neurons then helps, at least temporarily, diminish symptomatic trembling (Bauer et al., 2014).

While the pharmacological and surgical treatments for PD seek to relieve symptoms for as long as possible, evidence is mounting that the most useful treatment for prolonging life and reducing symptoms may be a series of broader lifestyle changes including diet, and cognitive and physical exercise (Khang, Park, & Shin, 2015; McNeely, Duncan, & Earhart, 2015; Mo, Hannan, & Renoir, 2015). Exercise has long been linked to beneficial cardiovascular effects, which can secondarily improve numerous PD symptoms, possibly owing to increased production of trophic factors within the central nervous system (Lowder, Padgett, & Woods, 2005; Passos, Ascensão, Martins, & Magalhães, 2015). While alone not as effective as medication in the symptomatic treatment of PD, exercise and associated life style changes have been demonstrated to produce improvements in non-motor symptoms of PD such as cognitive impairment and reduce inflammation which, potentially, may slow disease progression (Petzinger et al. 2013; Zou 2015; Cotman, Berchtold, and Christie 2007).

While our current techniques can temporarily eliminate PD symptoms, there is of yet no clear treatment option which can halt the neurodegeneration or permanently reduce motor or non-motor symptoms (PD MED Collaborative Group, 2014). Current research is focused on the identification of risk factors and biomarkers in order to allow diagnoses and treatment to begin in early and perhaps even prodromal stages of PD. Indeed, novel treatment options including neuroprotective compounds, specific kinase inhibitors and neurosurgical interventions may be most effective early in the disease process rather than at the point of typical diagnoses (Kavanagh, Doddareddy, & Kassiou, 2013; Rinne et al., 1998). It is our proposition that new treatments should target basic biological mechanisms and signalling cascades which give rise to the neurodegenerative processes. In this regard, accumulating evidence has implicated the neuroinflammatory system in PD and its various co-morbidities (Crowley, 2014a; McGeer & McGeer, 2004). Additionally, we believe that current animal models of PD need to be refined so as to more closely mimic the complex disease state observed in PD. Thus, the present thesis aims to: (1) Provide a comprehensive model of PD that addresses genetic and environmental aspects of the disease and (2) Assess the peripheral and central inflammatory factors that contribute to the loss of nigrostriatal DA neurons.

Animal research has resulted in four well established common toxin models emerging, involving administration of 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), lipopolysaccharide (LPS) or pesticide exposure (paraquat and rotenone). Although certain aspects of these models may differ, they collectively involve at least three common pro-death pathways: 1) Neuroinflammatory cascades, 2) The production of oxidative stress molecules and 3) Disruption of the mitochondrial respiratory chain. As an

example, all of the aforementioned toxicants inhibit function of Complex 1 of the mitochondrial respiratory chain (paraquat at albeit at higher doses than the others), which results in programmed cell death through the liberation of intracellular ROS generation (Li et al. 2003; Turrens, Alexandre, and Lehninger 1985). While these models induce PD-like in different processes, all four have been shown to induce chronic neuroinflammation to varying degrees. LPS, for example, is thought to induce DA death primarily through activation and recruitment of microglia and peripheral leukocytes while others such as paraquat are thought to directly mediate cell death through oxidative stress and secondarily, (chronically) by continued activation of immune responses (Biesmans et al., 2013; Mangano & Hayley, 2009)

1.2 Inflammation and PD

The immune system is primarily comprised of two components, the innate and the adaptive systems, which collectively orchestrate all host defences responses (Iwasaki & Medzhitov, 2010; Kumar, Kawai, & Akira, 2011). Of primary interest in PD pathology is the innate immune component which has been linked in many studies to non-specific neuroinflammation (Vercammen, Staal, & Beyaert, 2008). Given the constant immune challenges encountered daily, the blood brain barrier (BBB) serves as the general means of protecting the delicate neurons from any overzealous immune responses (Iwasaki & Medzhitov, 2010; Kumar et al., 2011; Pachter, de Vries, & Fabry, 2003). The innate immune system is a general, rapid, inflammatory response for the detection of foreign pathogens such as viruses, fungi and bacteria. Macrophages, eosinophils, basophils, mast cells and natural killer T cells (NK T cells) are the main cellular components of this system (Kumar et al., 2011). Many of these cells operate by the process of phagocytosis, wherein

they engulf microbes or infected cells and enzymatically degrade these or release oxidative radicals (respiratory burst) to break down the invader (Babior, Kipnes, & Curnutte, 1973; Drath & Karnovsky, 1975). In general terms, inflammation is characterized by swelling, heat, redness and pain which come about largely from changes in vascular networks that give rise to the mobilization and accumulation of leukocytes in the affected area. Although acute inflammation is strongly associated with discomfort, it has critically important benefits in removing damaged or infected cells and increases the rate of healing (McGeer & McGeer, 2004; Shacter & Weitzman, 2002; Haiyan Xu et al., 2003). That said, chronic inflammation is being increasingly implicated in a multitude of diseases, particularly owing to the liberation of oxidative stress factors which have been reported to be elevated in protracted inflammation (Crowley, 2014b; Wadley, Veldhuijzen Van Zanten, & Aldred, 2013).

Oxidative stress is the result of an imbalance between reactive oxygen species (ROS) to antioxidants (Babior et al., 1973; Hybertson, Gao, Bose, & McCord, 2011). ROS are reactive molecules containing oxygen with an unpaired electron, these “loose” electrons seek to react with and ultimately damage other molecules, such as DNA or cellular membranes (Babior et al., 1973). ROS can also initiate signalling cascades which lead to the production of a subset of glycoproteins known as pro-inflammatory cytokines (Dinarello, 2000). Under basal conditions, ROS are produced by phagotizing cells and balanced through anti-oxidative enzymes, including superoxide dismutase, catalase and glutathione peroxidase (Apel & Hirt, 2004). When free radicals exceed the antioxidant capacity of a cell, pro-death necrotic or apoptotic cascades ensue (Apel & Hirt, 2004; Finkel & Holbrook, 2000; Ott, Gogvadze, Orrenius, & Zhivotovsky, 2007). Such free radicals are a natural by-product of immune mediated phagocytosis of damaged cells, which

release hydrogen peroxide, superoxide, nitric acid and peroxynitrate at the site of inflammation (Babior et al., 1973). Importantly in cases of chronic inflammation, oxidative stress load can "spill over", such that neighbouring cells (neurons in PD) are detrimentally affected (Choi, Kim, Kim, & Choi, 2009; Majno & Joris, 1995). This in turn recruits more immune cells renewing and prolonging the inflammatory response (Dinarello, 2000). Thus, in the case of the CNS, this can be especially damaging given that neurons are, in the majority of cases, non-mitotic and hence not renewed in the adult CNS. Also, as will be discussed in detail in the next section, the CNS has its own specialized inflammatory cells, namely the microglia, which, although homologues to the peripheral macrophages, do have their own distinct physiological profile.

The CNS is considered an immune privileged organ and thus is typically isolated from the majority of immunological stress (Pachter et al., 2003). The BBB largely accomplishes this task by as acting a physical barrier, comprised of endothelial cell tight junctions, astrocytic end feet and pericytes which line the blood vessels to restrict passage of large and hydrophilic molecules (Abbott, Patabendige, Dolman, Yusof, & Begley, 2010; Abbott, Rönnbäck, & Hansson, 2006). Any pathogens that manage to breach the BBB are normally rapidly dealt with by the dedicated CNS immune cells, called microglia (Abbott et al., 2010; Hickey, 1991). Microglia are one of the main sources of CNS ROS and combine various aspects of both the innate and adaptive immune systems (Kloss, Bohatschek, Kreutzberg, & Raivich, 2001).

Microglia are essentially a specialized form of macrophage and like the latter cell, display substantial plasticity, being able to exist in several states ranging from a highly ramified "resting" state to a highly activated state characterized by an amoeboid shape (Kreutzberg, 1996). Ramified microglia have long thin projections and a small central soma and, unlike the activated

microglia, they exhibit little cell body movement. However, all microglia are constantly dis- and re-assembling their long sensory projections which are extremely well tuned to physiologic conditions (Boche, Perry, & Nicoll, 2013; Kreutzberg, 1996). When a ramified microglial cell detects alterations in the extracellular microenvironment it undergoes retraction of projections and phenotypic changes in its expression of inflammatory messengers (Dheen, Kaur, & Ling, 2007). Activated microglial cells then become mobilized and play two primary roles in immunomodulation; 1) microglia can act like macrophages, phagocytizing pathogens, lysing damaged/infected cells and presenting antigens to major histocompatability complexes (MHCs) for peripheral T cell recruitment (Stollg & Jander, 1999), and 2) Release pro-inflammatory factors and undergo rapid proliferation and secrete chemoattractant cytokines and other molecules (Rogove, Lu, & Tsirka, 2002).

In the CNS, both astrocytes and microglia have been shown to produce pro-inflammatory cytokines, which typically act to sustain or amplify immune responses. These include tumour necrosis factor alpha (TNF-α), interleukins, (IL) and interferons (IFN) (Biesmans et al., 2013; Konsman, Parnet, & Dantzer, 2002). These cytokines are thought to play a pivotal role in the transition of neural inflammation from acute to chronic by altering the permeability of the BBB and allowing circulating leukocytes to enter the brain parenchyma (Wolburg & Lippoldt, 2002). In virtually all animal models of PD, increases in microglial activation state and proliferation have been reported (Mount et al., 2007; Schapira & Jenner, 2011). Perhaps even more strikingly, a primate study demonstrated that systemic treatment with the toxin MPTP, which is the precursor to the potent oxidant MPP+ and is taken almost exclusively into dopamine cells through the dopamine transporter, early in life induced protracted microglial activation that was still evident

up to 14 years later (McGeer, Schwab, Parent, & Doudet, 2003). This intriguing finding raises the possibility that even brief toxicant exposure might have permanent neuroinflammatory consequences. Other research has reported that the microglial state may be far more transient under certain circumstances. For instance, in the case of exposure to the endotoxin, LPS, maximal microglial activation was observed after 2 days and then subsided over the ensuing weeks (Litteljohn, Mangano, et al., 2010; Sugama et al., 2003). The fact that microglial activation appears to occur prior to the death of DA neurons (which typically takes several weeks) highlight the growing awareness that inflammation could play a primary rather than simply secondary or compensatory role in PD manifestations (Nagatsu & Sawada, 2005). Of course, this does not preclude the possibility that inflammatory processes might also shape the evolution of the disease state. In fact, our own work supports the notion that inflammatory cytokines and microglia might produce some of the co-morbid features of PD, including cognitive and affective deficits (Litteljohn, Nelson, & Hayley, 2014; Litteljohn, Cummings, et al., 2010).

It is important to note that the DA neurons of the SNc appear to be particularly vulnerable to inflammatory processes. Indeed, Kim et al (2000) reported that while intra-SNc infusion of LPS caused marked neurodegeneration, no such effect was observed when the endotoxin (at the same dose) was infused into other brain regions (thalamus, hippocampus and cortex). Yet, it has been reported, that although neuronal loss is confined to the SNc and possibly locus coeruleus in PD, marked microglial activation is observed across much of the CNS (Halliday & McCann, 2010). The increased vulnerability of SNc neurons may be related to several risk factors; 1) The SNc has perhaps the greatest density of microglia (Lawson, Perry, Dri, & Gordon, 1990), 2) Dopamine itself may contribute to vulnerability by its high oxidative potential (through auto-oxidization

producing potent free radicals which irreversibly alters proteins; (Miyazaki and Asanuma 2008), 3) Although speculative, it has been reported that the locus coeruleus degenerates in tandem with the SNc and hence, its potentially beneficial SNc innervation is lost (Zarow et al. 2003; German et al. 1992).

1.3 Genetic Components of PD and LRRK2

Leucine-rich-repeat-kinase 2 (LRRK2) is a recently discovered gene, also known as PARK8, that has been strongly linked to several diseases including familial PD, Crohn's disease and even leprosy (Alcalay et al., 2014; Hoefkens et al., 2013; Wang et al., 2014a). It is thought that the critical factor linking LRRK2 to each of these disease is its involvement in common inflammatory processes (Hoefkens et al., 2013). Accordingly, the highest levels of LRRK2 are found in immune cells, particularly B-cells, granulocytes, dendritic cells and microglia, along with the structural aspects of several organs including the spleen, lungs and kidneys (Wu et al., 2009).

The LRRK2 protein is an unusually large member of the Ras of complex (ROC)- carboxy terminal of Roc (COR) superfamily, containing seven known regions and is most notably, the only known protein to have both functional kinase and GTPase sites (Li, Tan, and Yu 2014). The two areas of interest with regards to PD, are the GTPase and kinase domains, although some recent attention has addressed the importance of the Leucine-rich-repeat (LRR) region (Berwick & Harvey, 2012; Biosa et al., 2013; Tong et al., 2012). The majority of PD-linked mutations occur in these regions; although, various pathological mutations have been found to some extent in every domain of the protein (Li, Tan, and Yu 2014).

Both the kinase and GTPase LRRK2 domains are thought to selectively phosphorylate immune proteins. Further, one hypothesis even raises the possibility that the GTPase domain is required to auto-phosphorylate the kinase domain, thereby sequentially affecting protein activation (Biosa et al., 2013; Manzoni et al., 2013). Other researchers have gone further still and provided recent data indicating that LRRK2 might have protein signaling capabilities independent of phosphorylation status (Berwick & Harvey, 2012). These studies report that kinase activity may not be necessary for all immune functions, in that LRRK2 may act as a scaffolding protein (Berwick & Harvey, 2012). In this case, proteins would bind to LRRK2 at the LRR domain and would be allosterically modulated favoring an activation state (Berwick & Harvey, 2012; Skibinski, Nakamura, Cookson, & Finkbeiner, 2014). To date while some basic studies have supported this hypothesis only one substrate has been reported, Wnt, which plays a signalling role in the β-catenin destruction complex (Berwick & Harvey, 2012).

Among all the different LRRK2 mutations, the majority of PD studies have focused on the most common mutation, the glycine to serine substitution (G2019S) at the kinase domain. Indeed, this mutation is thought to be responsible for 2% of all cases of PD (Alcalay et al., 2014; Greggio, Civiero, Bisaglia, & Bubacco, 2012). Due to the conserved nature of the ROC-COR superfamily, LRRK2 and its homologues can be found in organisms all the way down to single-celled amoeba allowing for comparative between species studies (Li, Tan, and Yu 2014). Curiously, while structure and posited function are highly conserved, tissue expression is actually quite variable across model species. This divergent finding is largely thought to stem from large differences in promoter region found across species (West et al., 2014). Most notably, rodents have substantially reduced promoter regions within the nigrostriatal tract, compared to primates

(West et al., 2014). As a consequence, bacterial artificial chromosome (plasmid transformed) transgenic rodents must be used for viable LRRK2 expression.

Within the lungs, LRRK2 expression is observed in the alveolar type II cells, which mediate the interaction of the environment with the body by producing the surfactant, thereby allowing oxygen transport into the circulatory system (Herzig et al., 2011). Meanwhile LRRK2 in the spleen is primarily located in B-cells undergoing maturation, where it presumably mediates adaptive immunity through interactions with CD4+ T-cells and transition to plasma B-cells. In the kidney, it has been reported that LRRK2 modulates autophagy and lysosomal homeostasis (Gómez-Suaga, Fdez, Blanca Ramírez, & Hilfiker, 2012; Herzig et al., 2011). Moreover, LRRK2 is localized to very specific regions of the kidney, yet it appears to have widespread functions within this organ, involving the elimination of potential toxins (West, 2015).

Although the exact mechanism of LRRK2 at peripheral sites is not entirely known, transgenic and knockout (KO; mice with no appreciable LRRK2 protein expression) models are currently painting a picture of a highly complex role across different organs systems. In a recent study utilizing LRRK2 KO and LRRK2 kinase dead (mice with normal LRRK2 protein levels but no functional kinase activity) mice, Herzig *et al.* reported that by six weeks of age, KO mice showed lung and kidney cellular pathology, whereas the kinase-dead mutants displayed pathology restricted to the kidneys. These data strongly suggest that that both the actual levels of LRRK2 and its kinase activity are involved in organ-specific pathology (Herzig et al., 2011; Tong et al., 2012). Yet, it should be underscored that data are extremely scant with regards to *in vivo* mechanisms involved and further replication studies are required.

1.4 The role of LRRK2 in inflammatory models of PD

While a plethora of animal models have been utilized in PD research, there is yet to exist a single comprehensive, progressive model that replicates the human condition (Blesa, Phani, Jackson-Lewis, & Przedborski, 2012). Currently, the leading models are either inflammatory or genetically based, but fail to incorporate the fundamental fact that PD is a complex heterogeneous condition that stems from multiple environmental hits superimposed upon a genetic vulnerability (Alcalay et al., 2014). In this regard, we have previously focused extensively on immune and chemical insults as a means of shaping the evolution of PD (Mangano & Hayley, 2009). In the present thesis, we take the next step and utilize an inflammatory hit (bacterial endotoxin) model in the context of the G2019S LRRK2 genetic mutation. Essentially, we hypothesize that: 1. the augmented LRRK2 protein levels and 2. the increased kinase activity evident with this mutant will increase susceptibility to the inflammatory LPS challenge.

Our previous studies demonstrated that relatively low doses of the bacterial endotoxin, LPS, or viral mimic, Poly I:C, enhanced the neurotoxic effects of paraquat, such that a substantial number of DA producing neurons were destroyed and PD-like symptoms emerged (rigidity and reduced movement) (Mangano & Hayley, 2009). This augmented neurodegenerative response was observed when paraquat was administered at the time of maximal microglial activation (2 days following LPS), suggesting that the inflammatory sensitized microglia likely contributed to the degenerative effects of later paraquat exposure. Moreover, sensitized microglia were consistent with a so called “M1” or pro-inflammatory phenotype, showing profound morphological changes and greatly enhanced expression of the catalytic subunit of NADPH

oxidase, gp91, which is responsible for the release of superoxide (Mills, 2012). Importantly, although relatively high concentrations of LPS alone had neurodegenerative consequences on DA neurons, our studies involved concentrations of the endotoxin that alone activated microglia but had no effect upon DA neuronal survival.

Research thus far, albeit only one study, has revealed that LRRK2 KO surprisingly did not influence the number of functional DA neurons apparent under basal condition or in response to MPTP insult (Andres-Mateos et al., 2009). We postulate that while MPTP increases oxidative stress its effects on inflammatory parameters is indirect and that a more direct inflammatory "hit" is required to uncover the effects of LRRK2. In this regard, LPS acts as such a direct hit by stimulating toll-like receptor 4 (TLR4), giving rise to characteristic intra-cellular protein cascades modulated by myeloid differentiation primary response gene 88 (MYD88) and the NF-κB pathway (Kaltschmidt & Kaltschmidt, 2009). This pathway alters genetic expression such that immune cells produce pro-inflammatory cytokines chemokines and adhesion molecules (Lawrence, 2009). These signalling molecules have been shown to play a role in PD in primate models, indeed inhibition of the NF-κB pathway is protective to DA neurons (Flood et al., 2011). While NF-κB is currently under investigation, it is seen as the "master switch" in inflammation and no selective treatment has yet been uncovered (Flood et al., 2011).

To date, Moehle et al (2012) is the only published *in vivo* study that assessed brain, cell specific, LRRK2 levels. Importantly, they found the highest LRRK2 concentration in glial cells, and LRRK2+ microglia were most responsive to LPS challenge (Moehle et al., 2012). Further, blocking LRRK2 active signalling sites using a small molecule inhibitor prevented the elevations of TNF-α

and iNOS that were previously induced by LPS (West et al., 2014). In a separate study, G2019S transgenic rats were found to have increased basal iNOS levels and altered DA neuron phenotypes consistent with those observed in oxidative stress studies (Lee et al. 2015). Similarly, microglia isolated from R1441G LRRK2 mutant (which displayed enhanced basal LRRK2 levels) displayed enhanced pro-inflammatory responses (increased TNF- a, IL-1b, IL-6) to LPS, and supernatant from these cultures was toxic to primary neurons (Gillardon, Schmid, & Draheim, 2012). At a functional level, these R1441G LRRK2 mutant mice displayed enhanced motor deficits in response to the pesticide rotenone (Liu et al., 2014).

In the current study we aim to assess whether the G2019S mutation will modify the impact of central administration of LPS (at 2 and 10 ug doses, which alone should have sub-optimal and robust damaging effect on DA neurons, respectively). Besides the hypothesized sensitizing effect of G2019S on nigrostriatal microglia, we also predict that splenic and kidney macrophage responses will likewise be augmented by the LRRK2 mutation. Collectively, these peripheral and central inflammatory changes are expected to result in a loss of nigrostriatal TH+ neurons and concomitant deficits in motor activity.

2. Methods

2.1 Animals

B6.Cg-Tg(Lrrk2*G2019S)2Yue/J transgenic mice were purchased from Jackson Laboratories concurrently with C57Bl/6J mice at 6 weeks of age. All animals were fed Harlan Labs 2018 rodent chow and housed under normal lighting conditions. The female mice received male bedding to induce eustress 48 hours before being paired in harems of one male to three female

mice. 14 days later females were separated from males and monitored for births which occurred approximately 21 days after pairing. At 21 days of age male pups were ear punched for identification, were placed on Harlan labs 2014 diet and had tail snips taken for genotyping. Males were group housed with littermates and enrichment in individually ventilated cages until 10 weeks of age when they were transferred into an experimental room and single housed in microisolator cages with only a nestlet until the experiment began at 12 weeks of age.

DNA was extracted from 0.5cm long tail snips collected at 21 days of age using the Qiagen DNEasy blood and tissue kit. Extracted DNA was analyzed using a BioRad MyIQ qPCR machine and the recommended Jackson Labs protocol to separate wild type animals and heterozygous transgenics based on melt curve data. Pure wild type products resulted in melt peaks at 80°C and heterozygous and homozygous transgenic amplicon products resulted in melt peaks at 84°C. Animals where genotyping was not conclusive were retested and excluded.

Table 2.1 Experimental Treatment Groups

Treatment Group	Genotype	Infusion	Sacrifice
1	Wild Type	Saline	23 Days Post Infusion
2	Wild Type	2µg LPS	23 Days Post Infusion
3	Wild Type	10µg LPS	23 Days Post Infusion
4	G2019S Overexpresser	Saline	23 Days Post Infusion
5	G2019S Overexpresser	2µg LPS	23 Days Post Infusion
6	G2019S Overexpresser	10µg LPS	23 Days Post Infusion

2.2 Surgeries

At 3 months of age male animals underwent stereotaxic surgery. Animals were anesthetized with 5% isoflourane then subcutaneously administered 0.3mL of saline and 20mg/kg of the analgesic Tramadol. Animals were secured in the Kopf Instruments Model 940

stereotax frame using xylocaine (2% lidocaine hydrochloride topical anaesthetic, AstraZeneca) coated earbars. A small, 1cm incision was made in the centre of the skull and a 1.5mm diameter hole was drilled in the skull at x=14mm and y=314mm relative to bregma over the left hemisphere. A 22 gauge injector was used to infuse 2µL of either saline, 1µg/µL LPS or 5µg/µL LPS directly above the SNc 4mm below the surface of the skull. A Harvard Apparatus picopump 11 double syringe pump was used to ensure a constant infusion over 4 minutes. The injector was left in place for 5 minutes after the infusion to allow the LPS/Saline to absorb into the tissue before slowly being removed. The hole in the skull was filled with Bone Wax® before suturing. The animals received 100% oxygen for 15 seconds once the surgery was complete before being removed from the stereotax in order to speed recovery, they were then placed into recovery cages situated half-on half-off a 37°C circulating water heating pad. Animals were under anesthesia at 1.5% isoflourane for approximately 25 minutes each and monitored for signs of distress for one hour after surgery. Animals were given mashed 2014 diet and hydrogel for 4 days after surgery and 20mg/Kg Tramadol sub-cutaneously twice a day for 3 days following surgery.

2.3 Behaviours

Sickness Scoring and Weights

Animals were weighed each morning for the first 4 days after surgery as well as one, two and three weeks after the LPS infusion. Sickness scores were taken according to Table 1 twice a day for the first 3 days after surgery and then on days 4, 7, 14 and 21.

Table 2.2: Sickness scores were assigned after analysis of each animal for 2 minutes from Hayley et al., 1999

Score	Symptoms
1	Normal looking
2	Slight lethargy, ptosis (droopy eyes) or piloerection (puffy fur)
3	Very lethargic, ptosis and piloerection, curled body posture
4	Very sick appearance, ptosis, piloerection, curled body posture, difficulty breathing, and general nonresponsiveness

Catwalk

Prior to surgery animals were given three days of catwalk training to acclimatize them to the machine (Noldus Catwalk XT 8.1, Wageningen, Netherlands). During training animals were placed at one end of the catwalk in the dark and allowed to move back and forth across the catwalk until the camera detected three runs in which the animal completely traversed the camera's view range in less than 5.5 seconds with a speed variation of less than 100%. On the first day of training animals were allowed 5 minutes or three complete runs while on days two and three animals remained on the catwalk until they completed all three runs. Animals deemed non-compliant were sprayed with compressed air during training to induce them to cross the catwalk. A baseline was taken the day before surgery and animals were again run on the catwalk on days 4, 10, 16 and 20.

Micromax

Animal's home cages were placed into the micromax infared beam break apparatus (Accuscan Instruments, Columbus, OH, USA) on the nights of days 4, 9, 15 and 20. Nestlets were removed from the cages and animal's home cages were placed such that eight beams bisected their cage several hours prior to data. Data collection began at 8pm. Beam breaks were recorded continuously for 12 hours overnight giving a mean score of overall motor activity.

Rotarod

On day 14 animals began training on the rotarod, which consists of a rubber beam 30cm above the ground. The rotarod spins at either a fixed or variable speed, underneath which infrared beams which detect when an animal falls. Animals received two days of training, on the first they were placed onto the beam for 5 minutes at 12 rpm and replaced every time they fell within the 5 minutes. This was repeated one hour later and an hour after that for a total of three training sessions on day one. On day 15 animals received their second rotarod training. The speed was increased to 22rpm and all other aspects were repeated. Test day occurred on day 16 when the animals were placed on the rod for 3 sessions an hour apart in which the speed of the rod increased from 2rpm to 44rpm of the course of five minutes. The speed and time at which each animal fell was recorded. During analysis the lowest score for each animal will be dropped.

Perfusion

Animals were intraperitoneally administered 0.15mL of sodium pentobarbital and level of anesthesia determined by toe pinch. Blood was flushed using 5mL of saline through the left ventricle to the right atrium followed by 40mL of 4% paraformaldehyde to fix the tissueBrains

were extracted and placed into vials containing 4% paraformaldehyde on ice. 24 hours later the brains were transferred to 10% sucrose and then transferred to 30% sucrose 48 hours after sacrifice.

2.4 Tissue Processing

Immunohistochemistry:

Brains were flash frozen and sectioned on a Fisher cryostat. Striatum sections were collected at 40µm thick for optimal antibody penetrance while SNc sections were collected at 60µm (the minimum require thickness for stereology. All sections were collected into 0.1M PB, washed twice in 10mM phosphate buffered saline (PBS) for 5 minutes each and then transferred into 0.3% H_2O_2 for 30 minutes. Following this sections were rinsed 3x10 minutes in 10mM PBS and transferred into a 5% normal goat serum (NGS) blocking solution for one hour. Every second section of striatum and SnC were stained for CD68 as follows: Sections were incubated overnight in a 5% NGS solution with 200µLs of 1:2000 anti-CD68 (AbD Serotech, Oxford, England). The following day sections were washed 3x5 minutes in 10mM PBS and submerged in 200µLs of 1.6% NGS solution with 1:500 anti-rat-biotin (Santa Cruz, Texas, USA). The sections were again washed 3x5 minutes in 10mM PBS then placed into 200µLs of 1.6% NGS solution with 1:1000 streptavidin horseradish peroxide (Jackson Immunoresearch, Pennsylvania, USA). These sections were again washed through 3x5 minutes in 10mM PBS and then reacted with diaminobenzidine (DAB). After 10 minutes 30µL of a 0.3% H_2O_2 solution was added and reacted for a further 5 minutes before the sections were washed three additional times in 10mM PBS and mounted. The following day

sections were dehydrated through graded alcohols, cleared through three clearene washes and coverslipped with DPX xylene based mount. The above procedure was repeated for the striatal TH staining however the primary and secondary anti bodies used were mouse anti-TH (Immunostar, Wisconsin, USA) and biotinylated mouse (SigmaAldrich, Missouri, USA). The above procedure was also used to stain the SNc TH however after the mouse anti-TH a conjugate mouse-streptavidin antibody was used in a 1.6% NGS solution at 1:200 concentration for 4 hours.

Blood brain barrier permeability and vascular reorganization was assessed for the posterior hippocampal sections and immediately behind the SNc using a mouse anti-IgG to target all antibodies in the perfused tissue. Greater staining intensity is correlated with increased BBB permeability and vascular changes can be observed qualitatively by examining the size and shape of vessels in this same stain. 60µm thick sections were prepared as in the above stains however following the blocker they were placed in a 1.6% NGS solution at a 1:2000 concentration of Alexa fluor488 (Life Technologies, NY, USA) anti-mouse antibody overnight. The sections were then rinsed in PBS and coverslipped using flouromount (Sigma-Aldrich, Missouri, USA), care was taken to ensure the sections received no direct light once the antibody was applied.

Microglial Assessment:

Photomicrographs at 10x magnification were taken on a Nikon Eclipse 400 light microscope of sections prepared as above. Microglia were assessed by two blind, independent rates with an inter-rater reliability of >90% using the guidelines in Table 2.2. Three striatal sections and eight SNc sections were assessed for each animal and the average of the scores from both raters for all sections were used to determine microglial activation of each animal.

Table 2.3 Microglial rating scale (derived from Mangano & Hayley, 2009)

Score	Symptoms
0	All cells are highly ramified with long thin projections and faint staining
1	Less than 5 cells per slice are either amoeboid or have short, thickened projections
2	Most cells on each slice have short, thickened projections, some may be ramified or amoeboid
3	Majority of cells are amoeboid with the remainder having short, thickened projections

Stereology: For the substantia nigra, MBF Bioscience Stereo Investigator was used for unbiased stereological count using the optical fractionator probe. Every second section from Bregma -3.08 to Bregma -3.18 was collected and stained, count areas were defined by tracing the outline of TH+ cells from the SNc. Cells were counted at 63x magnification, and tissue thickness was manually measured at every grid site (90 µm by 90 µm, counting frame: 60 µm by 60 µm, guard height: 3 µm, optical dissector height: 15 µm). The neuronal cell count reported was the estimated population using number weighted thickness.

2.5 Statistics

Statistical Analysis

All data were analyzed by two-way ANOVA, followed by Fisher's planned comparisons ($P<0.05$) where appropriate. In some cases where we determined no statistical differences data were collapsed across treatment or genotype and one-way ANOVAs followed by Fisher's planned

comparisons were run. All statistical analysis was carried out utilizing the StatView (Version 5.0) statistical software package from the SAS Institute, Inc.

3. Results:

3.1 Genotyping Results

Animals were bred using mothers with a heterozygous G2019S mutations linked to a human LRRK2 promoter and WT fathers. These pairing resulted in only two outcomes either pups born were heterozygous or pups. Tails were extracted as detailed in the methods and realtime PCR was performed on the resultant extractions coupled with a melt-curve to produce images such as Figure 3.1 which allowed us to clearly determine contaminations (Indicated by the lack of a peak at 75°C), WT animals (Peaks at 78°C) and the heterozygous animals later used in the experiment (Peaks at 84°C).

Insert Figure 3.1

3.2 Behavioural Data

Sickness outcomes

For the first week, animals were scored twice a day on sickness and one a day weight calculated. A subset of time points are presented here for the sake of clarity. No differences were observed between the two LPS doses and so we collapsed across dose ($F_{1,31}$=3.23, p>0.05). As seen in Figure 3.2 following LPS infusion all LPS exposed animals had increased sickness scores ($F_{3,45}$=13.753, p<0.001), These sickness symptoms were reflected in altered nesting behaviour, lethargy, unkempt fur and ptosis; however, there were no significant genotype or interaction

effects ($F_{1,45}$=0.440, p>0.05 and $F_{1,45}$=0.440, p>0.05). Saline animals exhibited none of these traits and received the lowest possible score on the scale. There was a significant interaction between LPS Treatment x Time ($F_{3,135}$=12.190, p<0.001), wherein LPS treated animals showed the expected reduced sickness scores as time progressed.

Figure 3.3 illustrates the alterations in weight from baseline following surgery. Saline treated animals gained a small amount of weight following surgery, while both of the LPS infused groups lost substantial weight following surgery ($F_{1,32}$=21.932, p<0.001). In the subsequent recovery days, WT animals regained weight significantly faster than G2019S mutants ($F_{3,96}$=3.878, p=0.028), with all animals returning to or exceeding baseline weight after 5 days.

Insert Figures 3.2 and 3.3

Motor Behaviours

Animals were tested once a week in the micromax beam break apparatus and catwalk (as described in the methods section) in order to observe any deficits in home-cage activity or gait, respectively. Additionally, during the final week, animals completed the rotorod test to assess motor coordination. The micromax test revealed no significant differences during the 12 hour dark cycle with treatment or genotype (Figure 3.4 $F_{2,34}$=0.074, p>0.05 and $F_{1,34}$=0.015, p>0.05). Similarly, these findings were mirrored in the lack of effect with regards to rotorod performance (Figure 3.5 $F_{2,42}$=0.097, p>0.05 and $F_{1,42}$=0.557, p>0.05). However, catwalk analyses indicated that G2019S animals had significant basal alterations in measures of gait throughout the experiment.

Most relevant for our Parkinson's model is the hind-limb base of support (BOS), which is a measure of the stability and distance hind limbs are placed when walking (Figure 3.6). The G2019S animals had significantly lower BOS scores across all time-points (Figure 3.6) ($F_{1,43}$=35.9, p<0.001). These mutant animals also showed reduced stand index (the speed at which paws leave the glass surface) in the catwalk test ($F_{1,43}$=8.58, p<0.05). Surprisingly, the current LPS doses employed did not significantly affect catwalk performance on any measures.

Insert Figures 3.4, 3.5 and 3.6

3.4 Immunohistochemical outcomes

Striatum

Posterior striatal slices were separately stained for tyrosine hydroxylase and CD68 in order to assess dopamine neuron and microglial numbers, respectively. Densiometric analysis that was carried out on the striatal dopaminergic projections revealed no significant differences between the treatments or genotype (Figure 3.7; $F_{2,26}$=0.65, p>0.05 and $F_{1,26}$=0.79, p>0.05). Analysis of CD68 staining was completed by three independent blind raters with an inter-rater reliability of above 90%. Animals treated with 10µg of LPS had significantly higher CD68 rating scores than their saline or 2µg injected counterparts (Figure 3.8; $F_{2,26}$=7.73, p=0.002). Genotype had no effect on outcome, neither was there any interaction observed between genotype and treatment ($F_{1,26}$=1.08, p>0.05 and $F_{2,26}$=1.344, p>0.05, respectively).

Insert Figures 3.7 and 3.8

Substantia Nigra

Our stereological quantification procedures revealed a significant difference in the number of tyrosine hydroxylase neurons between treatments, wherein animals treated with 10µg of LPS had significantly less neurons, an approximate 35% loss (Figure 3.10; $F_{2,19}$=6.69, p=0.006). G2019S animals were found to basally have higher neuronal counts than their WT counterparts, however, no interaction between genotype and treatment was observed ($F_{1,19}$=5.71, p=0.027 and $F_{2,19}$=0.211, p>0.05 respectively).

CD68 analysis was completed by three independent blind raters with an inter-rater reliability of above 90%. The omnibus ANOVA for CD68 staining density by treatment just missed significance (Figure 3.9; $F_{2,21}$=2.86, p=0.07). No genotype effect was found, nor did there appear to be any interaction effect ($F_{1,21}$=1.773, p>0.05 and $F_{2,21}$=1.152, p>0.05, respectively).

Insert Figure 3.10

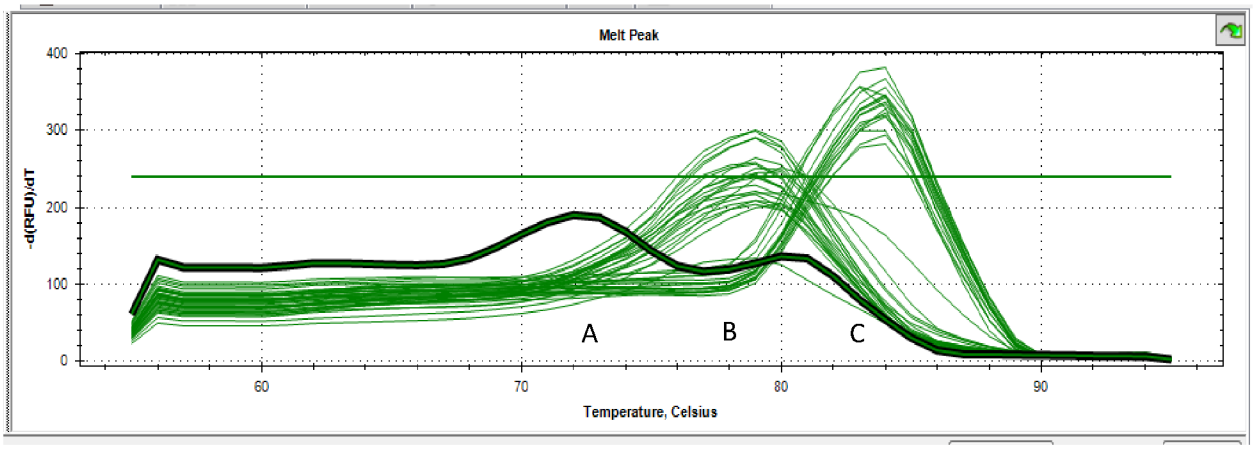


Figure: 3.1: Melt-curve generated following the qPCR protocol described above, the wide peak at A (73°C) is the non-template control and represents the melt-point of the primers, peaks at B (78°C) are wildtype samples and peaks forming at C (84°C) are heterozygous animals.

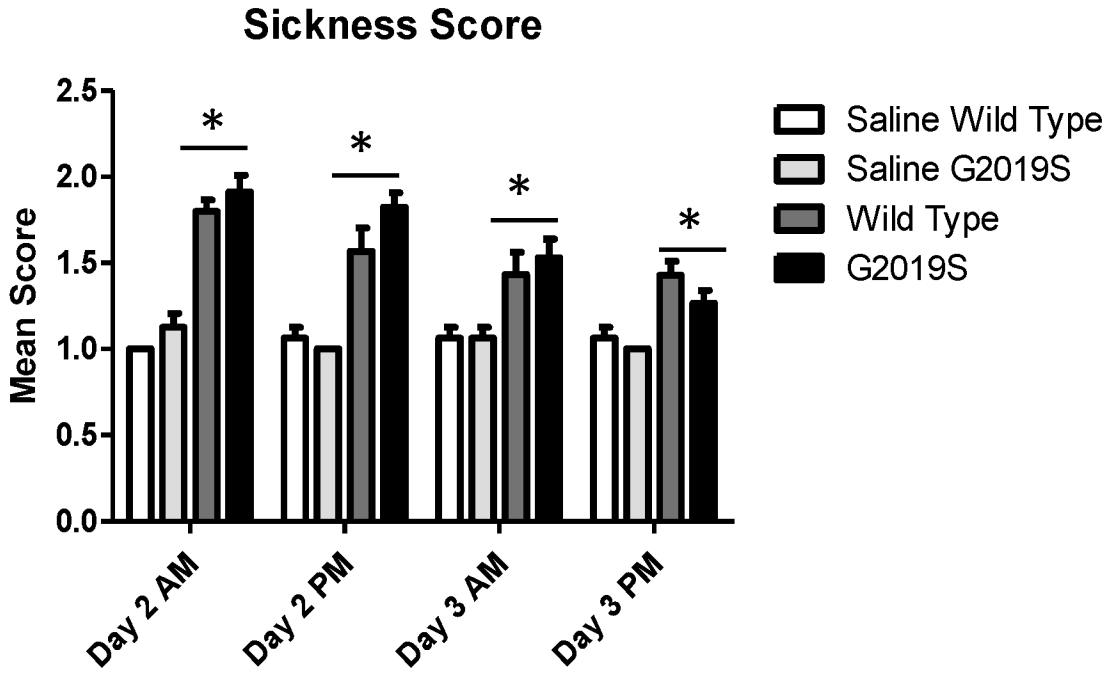


Figure 3.2: Scores were taken twice a day beginning 24 hours after surgery based on Table 2.2, control animals exhibited no sickness signs ($F_{1,16}$=0.214, p>0.05) while both G2019S and WT animals showed significant sickness symptoms lasting approximately 72 hours ($F_{3,45}$=13.577 ,p<0.001). * Represents a difference from Saline controls p<0.01

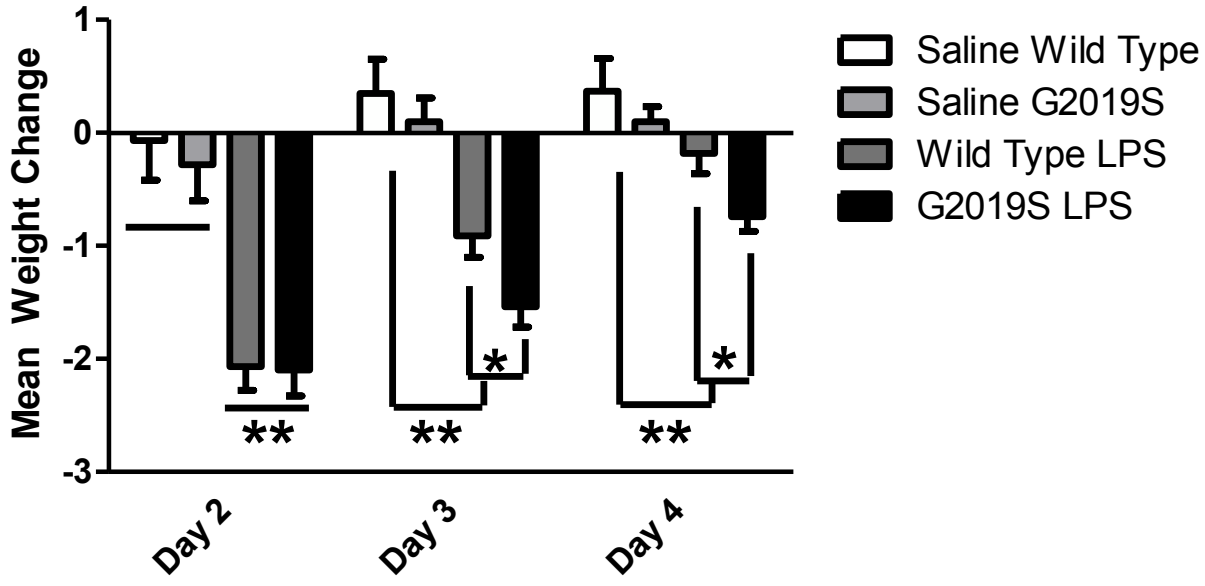


Figure 3.3: Weights were collected each morning for the first week following surgery, there were no differences between LPS treatments so scores were collapsed across dose. WT animals recovered significantly faster than G2019S animals although both were much lower than saline controls following surgery ($F_{3,96}$=3.878, p=0.028 and $F_{1,32}$=21.932, p<0.001. *p < 0.05, relative to WT LPS animals, **p < 0.01, relative saline controls

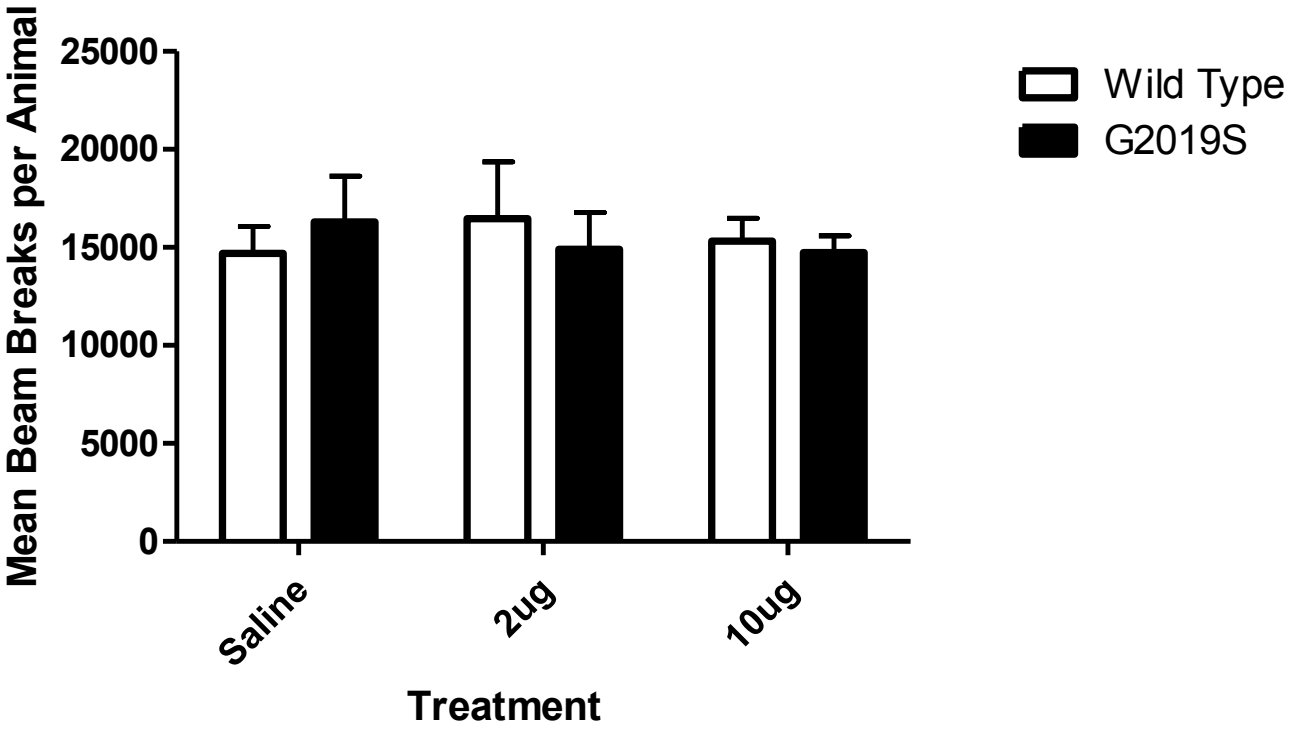


Figure 3.4: Mean beam breaks per animal over one 12 hour dark cycle three weeks following infusion. No significant differences were found between genotype ($F_{1,34}$=0.015, p>0.05) or treatment ($F_{2,34}$=0.074, p>0.05).

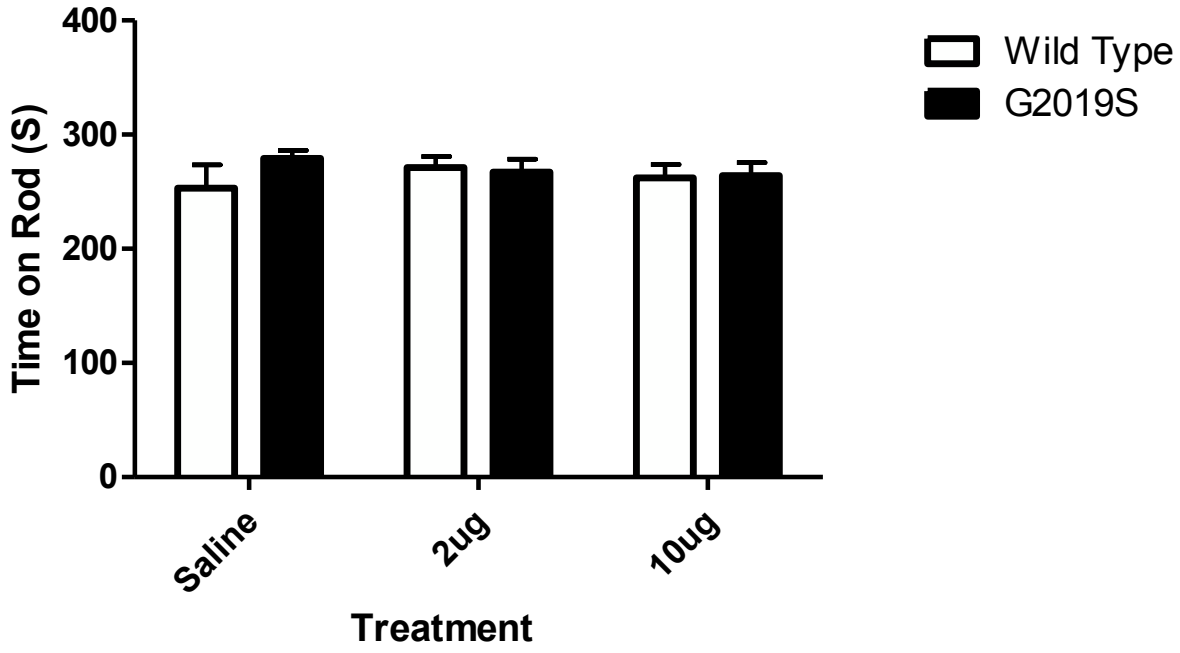


Figure 3.5: Average time spent on rotarod following three 5 minute trials three weeks post infusion, no genotype or treatment were found to differ significantly ($F_{2,42}$=0.097, p>0.05 and $F_{1,42}$=0.557, p>0.05)

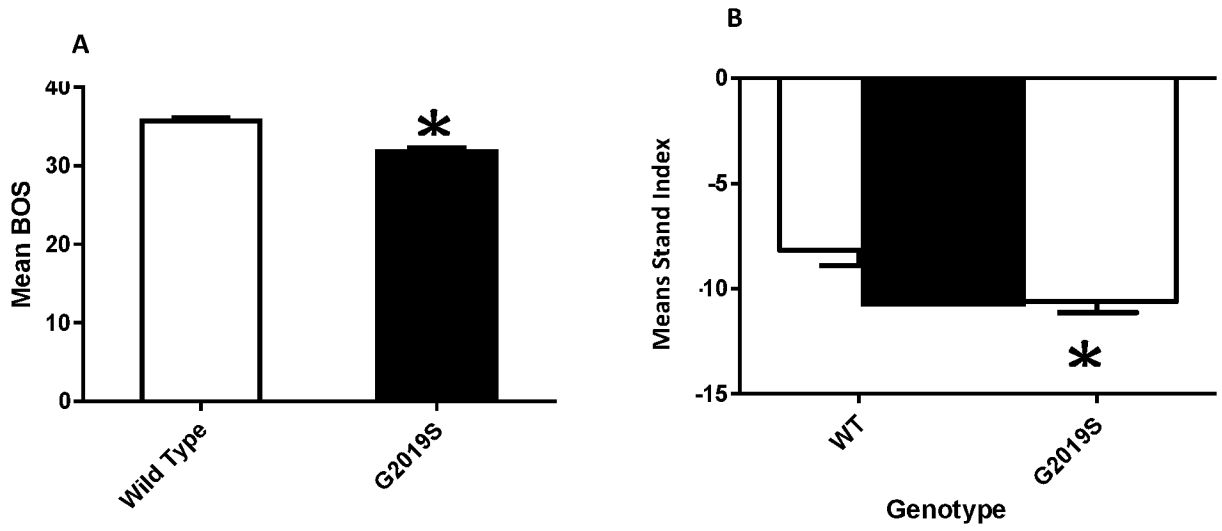


Figure 3.6: A: Base of support score generated from three runs completed on the Noldus Catwalk XT three weeks following infusion. B: Stand index scores taken from the same timepoint. There were no effects of LPS on either measure, however, G2019S overexpressors have a significantly reduced BOS and Stand index compared to WT animals ($F_{1,43}$=35.9, $p<0.001$ and $F_{1,43}$=8.58, $p<0.05$).

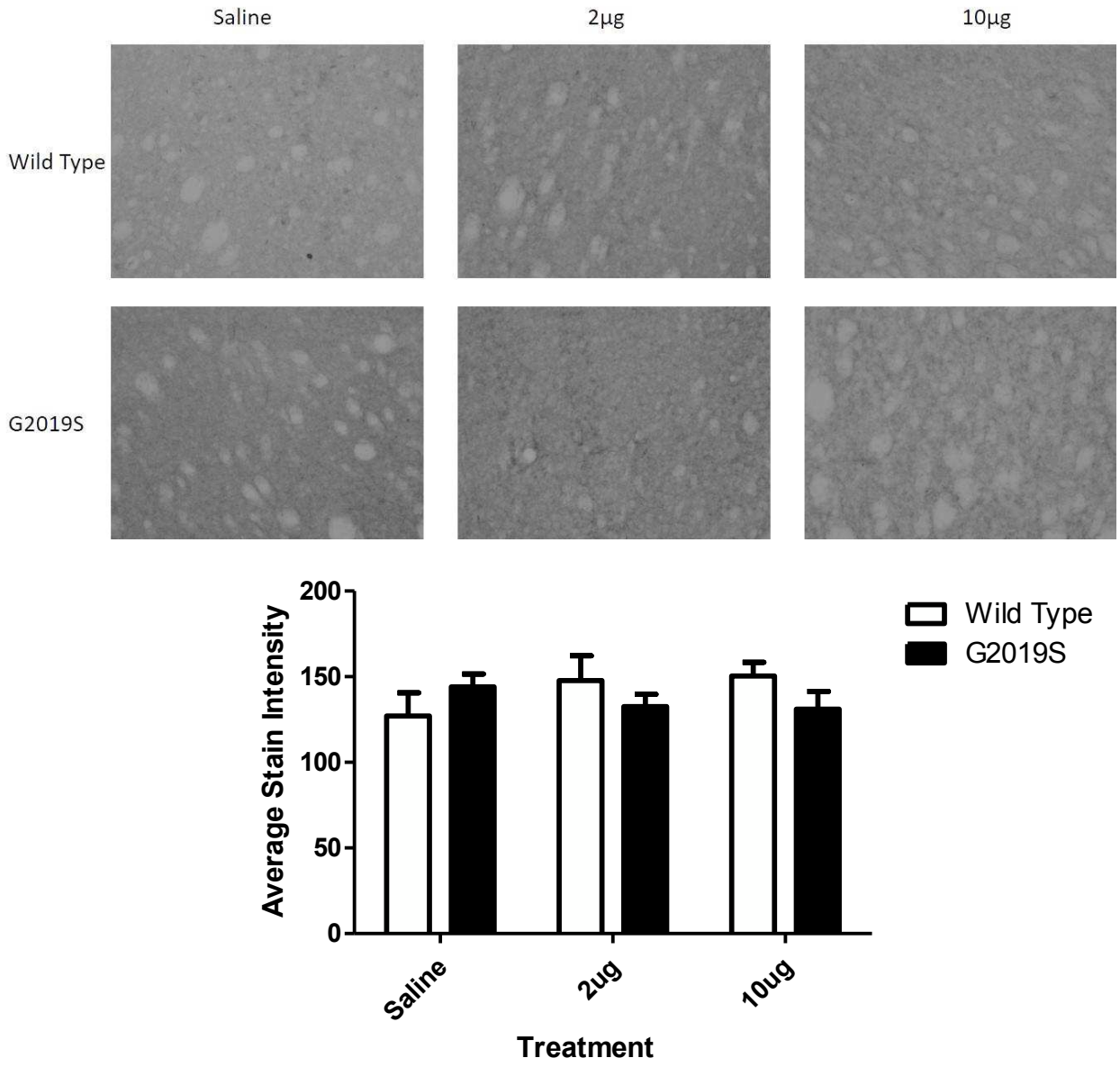


Figure 3.7: Photomicrographs taken at 20x magnification on a light microscope showing tyrosine hydroxylase positive neuronal projections in the striatum of wild type and G2019S animals. No significant differences were observed in staining intensities between any genotype or treatment ($F_{1,26}$=0.789, p>0.05 and $F_{2,26}$=0.651, p>0.05).

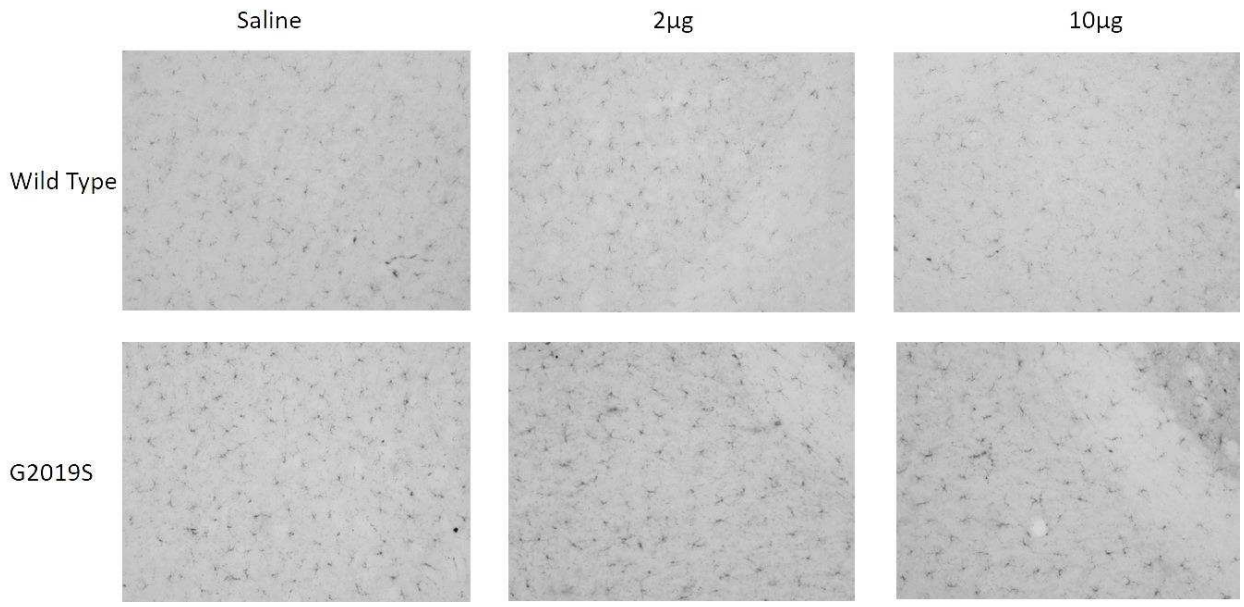
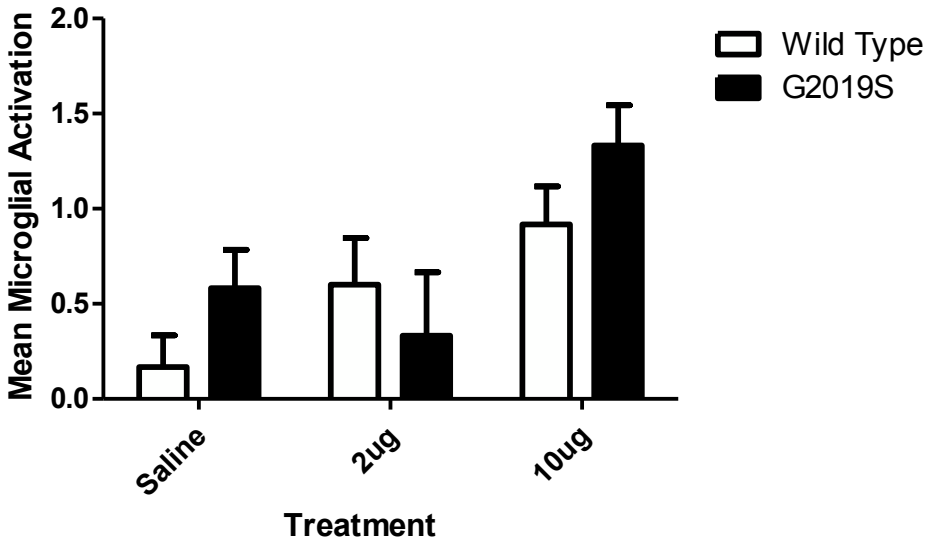


Figure 3.8:



Photomicrograph taken on a light microscope at 10x magnifications showing CD68+ staining in the striatum. Stains were evaluated based on Table 2.3; receiving higher scores for greater numbers of cells exhibiting shorter, thicker projections or amoeboid morphology. Microglial activation was found to be significantly increased in animals receiving 10ug of LPS ($F_{2,26}$=7.73, p=0.002), however no variance was observed by genotype ($F_{1,26}$=1.08, p>0.05) and there was no interaction between the two factors ($F_{2,26}$=1.344, p>0.05).

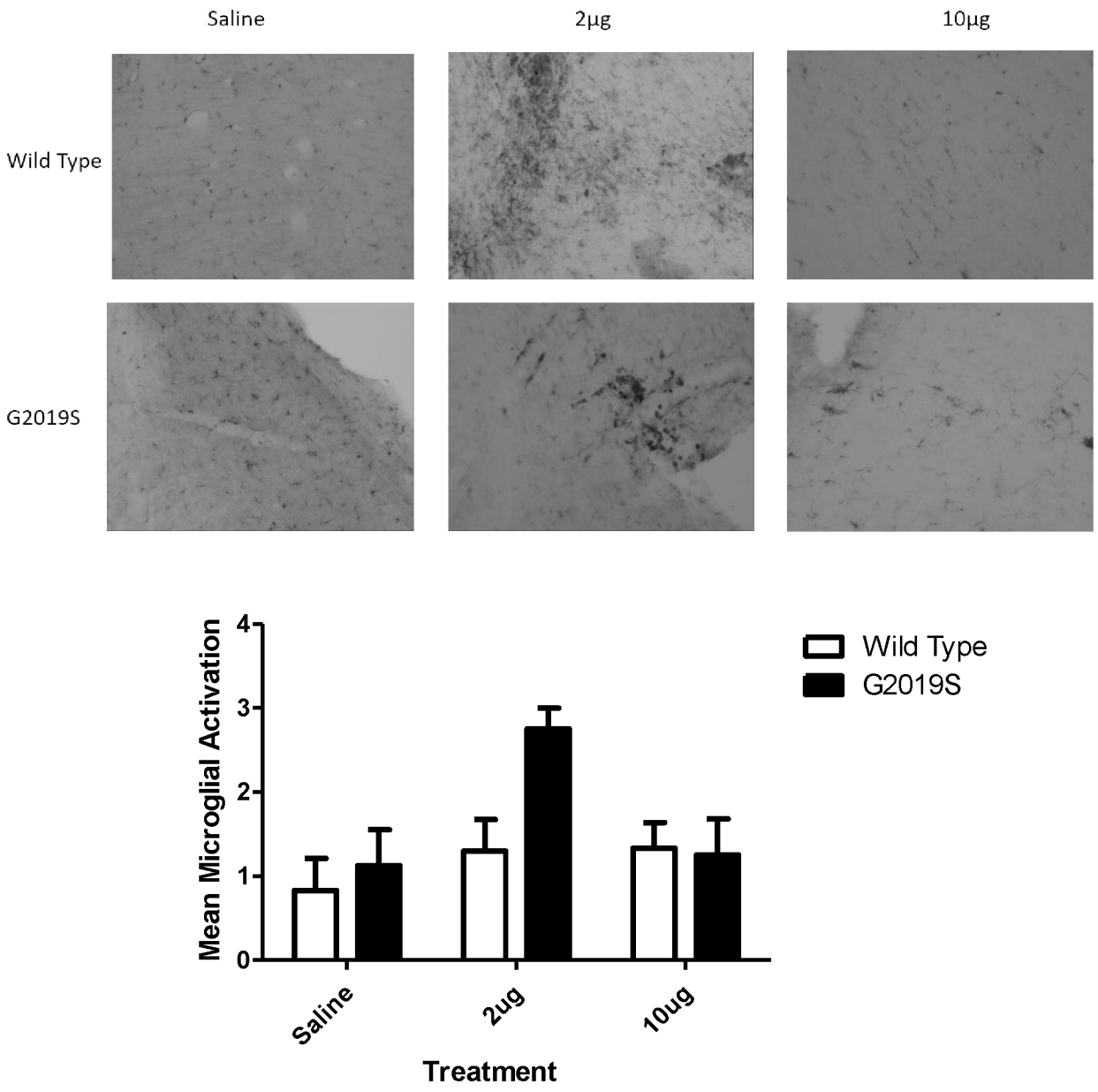


Figure 3.9: Photomicrographs illustrating the activation state of CD68+ cells within the SNc of animals in each treatment group with a graph illustrating the average activation scores found for each treatment group. Treatment with 2µg LPS trended to increase activation ($F_{2,21}$=2.87, p=0.07) however, genotype was not found to have an effect ($F_{1,21}$=2.59, p>0.05).

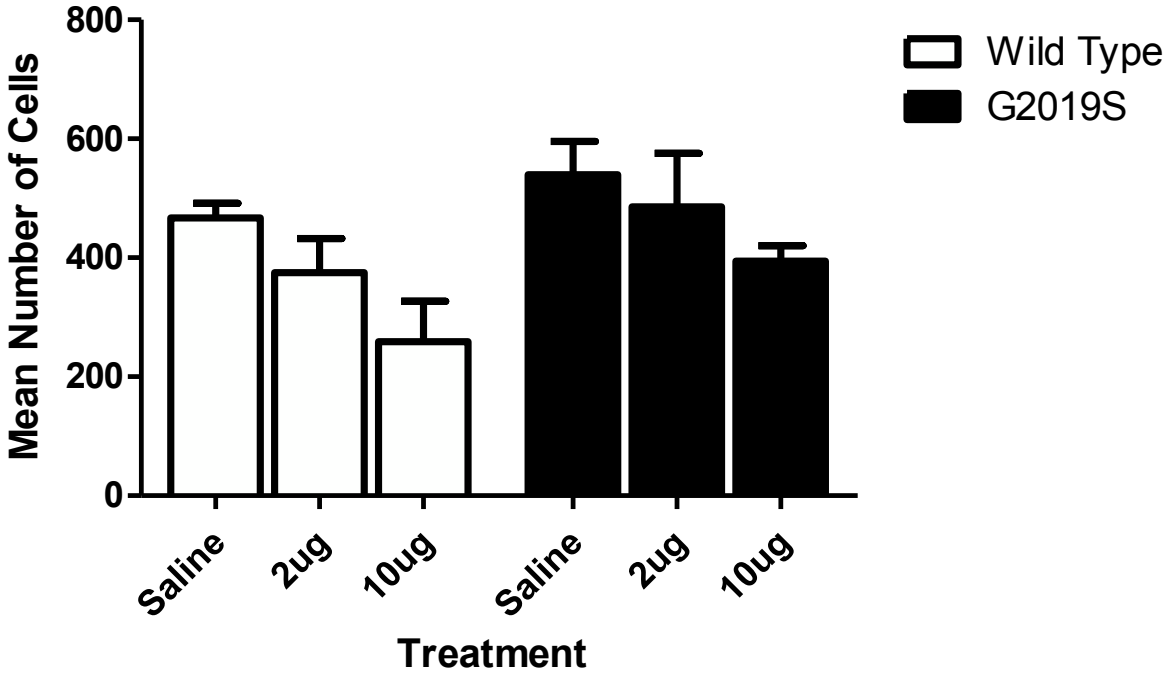


Figure 3.10: Graph representing the average number of TH+ cells found in the SNc of saline and LPS infused animals 21 days after exposure. Treatment with 10ug of LPS induced significant loss of TH+ neurons ($F_{2,19}$=6.691, p=0.006) while G2019S animals had higher neuronal counts ($F_{1,19}$=5.71, p=0.02), these effects showed no interaction ($F_{2,19}$=0.211, p>0.05).

4. Discussion:

Increasing evidence suggests that oxidative and immunological stressors are responsible for the loss of dopamine producing neurons in Parkinson's disease (PD) (Jenner & Olanow, 1996). Current data suggests that such oxidative factors primarily arise from: 1) disruption of mitochondrial function and 2) inflammatory responses within the central nervous system (CNS) (Crowley, 2014b; Ott et al., 2007). Epidemiologically, employees in industries exposed to environmental toxins, including welders and agricultural workers, are at significantly heightened risk of developing PD owing to the oxidative stress generated from these insults (Tanner et al., 2009; Teschke et al., 2014). There also exists support for the role of immunological challenges, including bacterial toxins and viruses, playing a role in the development of PD (Biesmans et al., 2013; Takahashi & Yamada, 1999). In fact, work environments correlated with high PD risk have also been linked to high ambient air levels of bacteria, such as agricultural centres (Rylander, 2002).

Immunological signalling induced by bacteria and environmental toxins can alter the homeostasis of the CNS through several mechanisms (Dupaul-Chicoine et al., 2013; Kreutzberg, 1996), most notably: 1) Activation of microglia which in turn leads to a pro-inflammatory cascade of cytokines (Nagatsu & Sawada, 2005) or 2) Disruption of the blood brain barrier (BBB), which allows circulating factors, cells and metabolites to enter the CNS (Pachter et al., 2003). These infiltrates can provoke oxidative stress and other pro-death mechanisms through the direct contribution of infiltrating peripheral immune cells and/or the activation of resident microglia (Dheen et al., 2007; Stollg & Jander, 1999). Additionally, both pathogenic and benign bacteria can elicit such changes through modulation of T_{reg} cells (ie gut microbiota) (Murai et al., 2009; Hui Xu

et al., 2008). Our own previous work has demonstrated that central LPS administration induced T cell infiltration and markedly stimulated microglial responses and the production of pro-apototic and oxidative species in the substantia nigra (SNc) (Blesa et al., 2012; Mangano & Hayley, 2009).

In this present study, we sought to elucidate the mechanism through which mutations in the LRRK2 gene contribute to PD pathology. Etiological studies have revealed that the LRRK2 gene contains the most common mutation found in patients with both familial and sporadic PD, Gly-2019-Ser (G2019S) (Gómez-Suaga et al., 2012; Greggio et al., 2012). We have attempted to modulate neuroinflammatory responses through the infusion of LPS above the SNc of both WT and G2019S animals. We hypothesized that the G2019S mutants would show an enhanced microglial reaction to the endotoxin, leading to dopaminergic neuronal death. As will be elaborated upon shortly, our findings generally did not support a substantive role for G2019S in modulating the impact of intra-SNc LPS infusion. These data are in agreement with recent findings showing that G2019S mutant mice were not different than their WT littermates in their responding to non-immunological insults (eg MPTP) (Andres-Mateos et al., 2009). However, it is important to note that the present study is the first to utilize an immune insult in the investigation of a LRRK2 mutant.

4.1 Immunological Involvement in PD

PD-like symptoms have been reported in an array of animal studies that assessed the impact of bacterial endotoxins, such as LPS, along with several epidemiological studies that have

linked bacterial and viral infections to a subset of PD cases (Conrady, Drevets, & Carr, 2010; Takahashi & Yamada, 1999). Several studies have demonstrated that viruses including HIV, poliovirus and herpes simplex can produce parkinsonian-like syndromes (Conrady et al., 2010) and some have postulated that early life viral infections may instigate disease later in life (Martyn, 1997; Takahashi & Yamada, 1999). It has also been posited that low dose exposures to immunogenic events such as these viruses or bacterial and chemical stimuli may induce mild, chronic neuroinflammation (McGeer et al., 2003). However, it is important to note that a single exposure to a viral or bacterial insult is unlikely to result in prolonged inflammation, nor is it likely to immediately alter the activation state of immune cells in the CNS (Biesmans et al., 2013). Similarly, despite the categorization of some populations as high risk for PD, notably welders and agricultural workers, the rates for these sub-populations are still quite low (Landrigan et al., 2005; Tanner et al., 2009) and it appears that cumulative environmental and immunological insults spread over the course of an individual's life may potentiate disease development.

Neuroinflammation has been demonstrated to increase circulating cytokine levels, positively bias microglial activation, and produce lasting vulnerabilities in dopamine producing neurons to low grade environmental challenges (McGeer & McGeer, 2004; Pachter et al., 2003; Stollg & Jander, 1999). In fact, animal studies using LPS injection(s) have demonstrated that very low doses administered intraperitoneally to pregnant dams greatly increase the vulnerability of their pups to further oxidative insults later in life (Xue et al., 2015). Moreover, work within our own lab has demonstrated that low level LPS exposures "prime" microglia, resulting in an enhanced release of toxic factors and augmented damage to SNc DA neurons when later exposed to environmental toxicants (paraquat) (Mangano & Hayley, 2009; Xue et al., 2015). Importantly,

within the CNS, the SNc (compared to the thalamus, cortex and hippocampus) was reported to be uniquely vulnerable to the effects of LPS(Kim et al., 2000). It was posited that the increased vulnerability SNc DA neurons to LPS stems from the greater density and reactivity of microglial present in this brain region (Kim et al., 2000) Further investigation into the vulnerability of the SNc has demonstrated that DA neurons in this region operate with oxidative stress levels far above comparable cells in other tissue, it is thought that the neurotransmitter dopamine and its auto-oxidative properties may account for this (Miyazaki & Asanuma, 2008).

The present study used a relatively higher dose of LPS (10µg single infusion) than previously used in our laboratory (2 ug; Mangano & Hayley 2009), in order to activate microglia to a level sufficient to induce neurodegeneration in the absence of any other challenge. To this end, although LPS treatment in WT mice had no effect on the activation state of SNc microglia (as indicated by CD68+ staining), G2019S mice did display (at least at the 2 ug dose) increased CD68 nigral staining (Figure 3.11, trend p=0.07). It is unclear why the 2 ug dose was more potent than the 10 ug LPS dose, but may be related to the temporal dynamics of the microglial response or the fact that the 10 ug dose actually provoked compensatory "recovery" process in the microglia. The most important point is that the microglial activation effect seemed to be restricted to the G2019S mice, supporting the contention that LRRK2 is an important regulatory of neuroinflammatory responses. Within the striatum we observed a separate effect, both G2019S and WT mice showed increased microglial activity at the 10µg dose but not the 2µg dose. The striatum is located further from the site of injection than the SNc and thus these alterations are likely due to microglia responding to the degeneration of TH+ projections originating from SNc neurons which were lost following LPS infusion. We would expect greater microglial

activation with appreciable striatal DA terminal loss thus this finding makes sense in the context of our TH+ cell counts.

Some toxicant models (ie 6-OHDA) are applied to the striatal dopamine terminals and their upstream effects are monitored. We selected the SNc as our primary site of interest following the work of Kim *et al.,* 2000 described above that demonstrated the most robust microglial populations in this brain region. The most likely reasons for the relatively minor microglial changes in wild type mice compared to our previous studies, is the timing of sacrifice relative to the LPS infusion. In this current study we were interested primarily in examining the end products of LPS mediated neuroinflammation; SNc DA loss and motor impairment (Choi et al., 2009; Sharma & Nehru, 2015). As such, we sacrificed our animals 24 days following LPS infusion as opposed to the 2-7 days used in our earlier work which found that LPS infusion resulted in microglial activation and pro-inflammatory signalling (Litteljohn, Mangano, et al., 2010; Mangano & Hayley, 2009). Indeed, it has been reported that early degeneration following SNc LPS infusions can begin in as little as three days but is not quantifiable until 14 days (5% loss) and is complete between 21 and 30 days (Li, Sun, Cao, Zhong, & Tong, 2004). Accordingly, we did find that LPS induced a dose-dependent loss of SNc DA neurons, however, as previously referenced, the G2019S genotype did not appear to alter this effect.

CD68 is a glycoprotein typically bound to low density lipoprotein (Holness & Simmons, 1993) and is found on macrophages, monocytes and microglia (Holness & Simmons, 1993). While CD68 is not itself an indicator of microglial activation, it is expressed on both ramified and amoeboid microglia (Holness & Simmons, 1993), allowing for the characterization of morphology (activated cells are amoeboid and compact in morphology while “resting” ramified microglia have

long thin projections and a small cell body) and hence, activation state indirectly (Nemenova, Turbina, Novikova, & Faĭnshte in, 1976). CD68 has one major drawback, it cannot distinguish between resident amoeboid microglia and infiltrating peripheral immune cells such as macrophages (Williamson, Sykes, & Stohlman, 1991). Hence, future studies will analyze the integrity of the BBB and use antibodies specific to certain peripheral immune cells in order to determine the presence and extent of infiltrating immune cells (Skulina et al., 2004; Williamson et al., 1991).

4.2 LRRK2 G2019S involvement in PD

Current research has focused on determining the role that LRRK2, specifically the G2019S and R1441G/C mutations, plays in the development of PD (Gillardon et al., 2012; Lee, Tapias, Di Maio, Greenamyre, & Cannon, 2015b). Besides PD, mutations in the LRRK2 gene have been strongly associated with risk of developing several inflammatory diseases notable Crohn's and leprosy (Hoefkens et al., 2013; J.-Q. Li et al., 2014a; D. Wang et al., 2014b). This coupled with genetic expression profiles of LRRK2 (It is found most strongly in immune cells) suggested that LRRK2 may be linked to inflammatory processes (Greggio et al., 2012; Skibinski et al., 2014). Human genome sequencing has demonstrated that different mutations of LRRK2 are responsible for the increased risk of these other inflammatory diseases; although the majority of these mutations appear to involve the GTPase and kinase domains of the protein which have been investigated in auto-phosphorylation and autophagy activation (Greggio et al., 2012; Herzig et al., 2011; Reynolds, Doggett, Riddle, Lebakken, & Nichols, 2014). Several mutations have even been located which are thought to be protective; however, there is currently little information

available on the effects or mechanisms of these mutations (Giesert et al., 2013; Heckman et al., 2014; Reynolds et al., 2014; Wu et al., 2013).

While the mechanism through which LRRK2 acts is still largely a mystery most literature supports three major alterations in carriers of the mutation 1) decreased neural autophagy and macroautophagy (Manzoni et al., 2013) 2) increased immune proliferation (Gillardon et al., 2012) and 3) increased response to immune challenges (Gillardon et al., 2012). In the current study, we assessed microglial activation as pertains to loss of DA neurons and our next step (future study) will involve the assessment of markers of autophagy (eg LC3) and oxidative stress (eg GP91) to examine effects of the LRRK2 G2019S mutation in a more mechanistic fashion. Indeed, we previously found GP91 to be robustly up-regulated within specific microglial cells activated by immune (LPS, poly I:C) challenges and other researcher supports a correlational link between GP91 expression and oxidative stress in the CNS (Bobyn et al., 2012; Litteljohn, Mangano, et al., 2010; Vaziri, Dicus, Ho, Boroujerdi-Rad, & Sindhu, 2003).

The effects of the G2019S mutation appear to be strongly correlated with age. Indeed, phenotypic differences in mice have not been reported before 6 months of age, whereas differences in motor behaviours, autophagy and oxidative stress continue to accumulate until at least 14 months of age (Chou et al., 2014; Lee et al., 2015b; Saha et al., 2015). We found that our G2019S mutant animals exhibited altered gait phenotypes prior to and irrespective of LPS treatment (Figure 3.7). Using the catwalk apparatus, G2019S animals at 3 months of age were found to display significantly narrower hind limb base of support and lowered stand index. The catwalk is a highly sensitive instrument specifically designed to detect gait deficits in PD and stroke animal models (Wang et al., 2012). So far, no studies utilizing the catwalk in the context

of LRRK2 mutations have been reported; which may be related to time of testing since deficits might not appear until animals become of advanced age (Lee et al., 2015a). We found no alterations in gross locomotor activity or coordination (micromax and rotarod), these findings were not totally un-expected as Figure 3.10 illustrates there was probably not a sufficient loss of striatal dopamine projections to induce overt motor deficits. Future studies investigating neuroinflammatory processes in LRRK2 animals would benefit from utilizing aged animals and assessment of behaviour at multiple time points in order to capture any transient vs permanent deficits.

4.3 Neuroinflammatory and neurodegenerative effects of LPS

LPS administered peripherally provokes a near immediate neutrophilic infiltration, followed by mobilizing macrophages and monocytes within 24 to 48 hours of the initial insult (Gabay, 2006; Kloss et al., 2001; Sharma & Nehru, 2015). This mobilization typically results in the release of pro-inflammatory cytokines that further increase the immune response. These cytokines, barring further stimuli, are soon balanced by later released anti-inflammatory cytokines (Konsman et al., 2002; Lawrence, 2009). Interestingly, researchers recently discovered that low doses of peripheral LPS not only activate peripheral immune cells and the microglia of the CNS, but induce the microglia to physically engulf axon terminals approximately four days after administration (Chen et al., 2012). Furthermore, previous data has found that LPS "priming" is temporally sensitive, with Mangano *et al.* 2009 finding paraquat administered 48 hours after LPS infusion resulted in synergistic pro-inflammatory effects and DA cell loss while paraquat administered 7 days following LPS was neuroprotective compared to paraquat treated animals.

The LPS model of PD has been used most extensively in rats (Daher et al., 2015; Kim et al., 2000; Lee et al., 2015b; Sharma & Nehru, 2015); results have been found to be dramatic and consistent across studies. In contrast, infusions in mice often require higher doses of LPS and show variable responses (Hunter et al., 2009; Tanaka et al., 2013). For instance, Sharma & Nehru found in 2015 that administration of 5ug of LPS in the SNc of 7 week old Sprague-Dawley rats produced increasing weight loss over several weeks, coupled with deficits in several aspects of motor activity. These same animals displayed a 60% decrease in TH neurons in the SNc. In contrast, mouse models struggle to obtain 40% TH+ cell loss even at doses exceeding 20µg of LPS (Hunter et al., 2009; Tanaka et al., 2013).

At the time of study conception, no LRRK2 mutant rats had been generated, our next follow-up studies will utilize a dual hit LPS followed by paraquat injection regimen in order to induce more profound inflammatory and degenerative effects in order to replicate previous mouse studies in WT animals which found more profound neuroinflammatory and degenerative effects. We currently used LPS alone as: 1) We hypothesizing that G2019S mutation would greatly increase vulnerability to LPS, 2) Breeding and genotyping the mutants has been extremely time consuming (thus we did not want too many experimental groups (lowering power) and 3) We wanted to first establish and dose-dependent effects of LPS prior to the introduction of a second toxicant hit.

4.4 Neuroinflammation, the G2019S mutation and oxidative stress

In order to assess the overall "clinical" state of animals we assessed sickness and weight scores. Although general sickness ratings were markedly increased by LPS, the mutation did not further modify this effect. However, we did determine that G2019S animals recovered weight significantly slower than WT animals (Figure 3.3). The fact that no vehicle treated animals demonstrated any weight loss or sickness alterations confirms that these were LPS effects and not due to any lingering malaise from surgery. These data suggest that the G2019S mutation may not necessarily intensify the severity of an initial immunological challenge, but might prolong the "window" of recovery.

While much research has focused on the role of LRRK2 in immune cells, other lines of investigation have examined the role of LRRK2 in neurons and astroglia which express a truncated form of the protein (Giesert et al., 2013; Saha et al., 2015). Interestingly, these studies have found age-related dysfunction of autophagy localized to dopamine neurons (Saha et al., 2015). As neurons age, animals with the LRRK2 G2019S mutation lose dopamine neuron autophagic functions. In the same study, α-synuclien was co-expressed and found to have synergistic dysregulatory effects on autophagy. A second study found pharmacological inhibition of LRRK2 G2019S proteins in transgenic rats was strongly protective in the face of α-synuclien related neuroinflammation and cell death (Daher et al., 2015). These findings suggest that alterations in the immune cells themselves may not be responsible for the greater prevalence of PD in LRRK2 G2019S carriers, instead improper clearance of protein aggregations and damaged organelles

accumulating with age may induce a more vulnerable state in SNc DA neurons to oxidative stressors.

4.5 Conclusions

Within this study, we have examined the effects of two doses of LPS infused into the SNc of WT and LRRK2 G2019S overexpressing mutant mice. Briefly, we found that the G2019S over-expressors exhibited a differential basal motor phenotype on the catwalk apparatus from WT littermates. Our sickness and weight data collected in the first week following surgery also suggested that the G2019S mutation may play in the "clinical" recovery phase from immune challenges. The loss of TH+ neurons in the SNc suggested that genotype did not modify the neurodegenerative impact of LPS alone at this timepoint. Also, our limited evidence of a microglial response at 24 days support the theory that multiple environmental challenges may be required to fully realize such outcomes. Future studies will focus on three key areas related to the involvement of the G2019S mutation in neuroinflammatory processes. Firstly, (a) given the increasing evidence supporting increasing age with behaviour and immunological changes in naïve G2019S animals we will explore the relationship between age and toxicant exposure in G2019S animals, secondly (b) we will infuse LPS above the SNc, however this infusion will be followed by peripheral administration of paraquat in order to determine whether the LPS may prime G2019S mutant immune cells. Our final (c) study will examine whether G2019S mutants have a longer window of sensitivity to paraquat treatment following LPS infusion.

5. References

Abbott, N. J., Patabendige, A. A. K., Dolman, D. E. M., Yusof, S. R., & Begley, D. J. (2010). Structure and function of the blood-brain barrier. *Neurobiology of Disease*. http://doi.org/10.1016/j.nbd.2009.07.030

Abbott, N. J., Rönnbäck, L., & Hansson, E. (2006). Astrocyte-endothelial interactions at the blood-brain barrier. *Nature Reviews. Neuroscience*, *7*(1), 41–53. http://doi.org/10.1038/nrn1824

Abboud, H., Floden, D., Thompson, N. R., Genc, G., Oravivattanakul, S., Alsallom, F., … Fernandez, H. H. (2015). Impact of mild cognitive impairment on outcome following deep brain stimulation surgery for Parkinson's disease. *Parkinsonism & Related Disorders*, *21*(3), 249–253. http://doi.org/10.1016/j.parkreldis.2014.12.018

Alcalay, R. N., Mirelman, A., Saunders-pullman, R., Tang, M., Santana, H. M., Raymond, D., … Levy, O. (2014). NIH Public Access, *28*(14), 1–12. http://doi.org/10.1002/mds.25647.Parkinson

Anderson, K. E. (2004). Behavioral disturbances in Parkinson's disease. *Dialogues in Clinical Neuroscience*, *6*(3), 323–332.

Andres-Mateos, E., Mejias, R., Sasaki, M., Li, X., Lin, B. M., Biskup, S., … Dawson, V. L. (2009). Unexpected lack of hypersensitivity in LRRK2 knock-out mice to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *29*(50), 15846–15850. http://doi.org/10.1523/JNEUROSCI.4357-09.2009

Apel, K., & Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology*, *55*, 373–399. http://doi.org/10.1146/annurev.arplant.55.031903.141701

Baba, Y., Higuchi, M. A., Abe, H., Fukuyama, K., Onozawa, R., Uehara, Y., … Yamada, T. (2012). Anti-cholinergics for axial symptoms in Parkinson's disease after subthalamic stimulation. *Clinical Neurology and Neurosurgery*, *114*(10), 1308–1311. http://doi.org/10.1016/j.clineuro.2012.03.046

Babior, B. M., Kipnes, R. S., & Curnutte, J. T. (1973). Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *Journal of Clinical Investigation*, *52*(3), 741–744. http://doi.org/10.1172/JCI107236

Barrett, M. J., Hac, N. E., Yan, G., Harrison, M. B., & Wooten, G. F. (2015). Relationship of age of onset and family history in Parkinson disease. *Movement Disorders*, *00*(00), n/a–n/a. http://doi.org/10.1002/mds.26166

Bauer, R., Martin, E., Haegele-Link, S., Kaegi, G., von Specht, M., & Werner, B. (2014). Noninvasive functional neurosurgery using transcranial MR imaging-guided focused ultrasound. *Parkinsonism and Related Disorders*, *20*(SUPPL.1), S197–S199. http://doi.org/10.1016/S1353-8020(13)70046-4

Bernheimer, H., Birkmayer, W., Hornykiewicz, O., Jellinger, K., & Seitelberger, F. (1973). Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical

correlations. *Journal of the Neurological Sciences*, *20*(4), 415–455. http://doi.org/10.1016/0022-510X(73)90175-5

Berwick, D. C., & Harvey, K. (2012). LRRK2 functions as a wnt signaling scaffold, bridging cytosolic proteins and membrane-localized LRP6. *Human Molecular Genetics*, *21*(22), 4966–4979. http://doi.org/10.1093/hmg/dds342

Biesmans, S., Meert, T. F., Bouwknecht, J. a., Acton, P. D., Davoodi, N., De Haes, P., … Nuydens, R. (2013). Systemic immune activation leads to neuroinflammation and sickness behavior in mice. *Mediators of Inflammation*, *2013*. http://doi.org/10.1155/2013/271359

Biosa, A., Trancikova, A., Civiero, L., Glauser, L., Bubacco, L., Greggio, E., & Moore, D. J. (2013). GTPase activity regulates kinase activity and cellular phenotypes of parkinson's disease-associated LRRK2. *Human Molecular Genetics*, *22*(6), 1140–1156. http://doi.org/10.1093/hmg/dds522

Blesa, J., Phani, S., Jackson-Lewis, V., & Przedborski, S. (2012). Classic and new animal models of Parkinson's disease. *Journal of Biomedicine and Biotechnology*. http://doi.org/10.1155/2012/845618

Bobyn, J., Mangano, E. N., Gandhi, A., Nelson, E., Moloney, K., Clarke, M., & Hayley, S. (2012). Viral-toxin interactions and Parkinson's disease: poly(I:C) priming enhanced the neurodegenerative effects of paraquat. *Journal of Neuroinflammation*, *9*(1), 86. http://doi.org/10.1186/1742-2094-9-86

Boche, D., Perry, V. H., & Nicoll, J. A. R. (2013). Review: Activation patterns of microglia and their identification in the human brain. *Neuropathology and Applied Neurobiology*. http://doi.org/10.1111/nan.12011

Bötzel, K., Ph, D., Daniels, C., Deutschländer, A., Gruber, D., Hamel, W., … Reu, A. (2006). A Randomized Trial of Deep-Brain Stimulation for Parkinson's Disease.

CD., B. S. J. P. M. (1993). Suppressive effect of L-dopa on dopamine cells remaining in the ventral tegmental area of rats previously exposed to the neurotoxin 6-hydroxydopamine. *Movement Disorders : Official Journal of the Movement Disorder Society*, *8*(2), 129–33.

Chaudhuri, K. R., Healy, D. G., & Schapira, A. H. V. (2006). Non-motor symptoms of Parkinson's disease: Diagnosis and management. *Lancet Neurology*. http://doi.org/10.1016/S1474-4422(06)70373-8

Chen, Z., Jalabi, W., Shpargel, K. B., Farabaugh, K. T., Dutta, R., Yin, X., … Trapp, B. D. (2012). Lipopolysaccharide-Induced Microglial Activation and Neuroprotection against Experimental Brain Injury Is Independent of Hematogenous TLR4. *Journal of Neuroscience*, *32*(34), 11706–11715. http://doi.org/10.1523/JNEUROSCI.0730-12.2012

Choi, K., Kim, J., Kim, G. W., & Choi, C. (2009). Oxidative stress-induced necrotic cell death via mitochondira-dependent burst of reactive oxygen species. *Current Neurovascular Research*, *6*(4), 213–222. http://doi.org/10.2174/156720209789630375

Chou, J.-S., Chen, C.-Y., Chen, Y.-L., Weng, Y.-H., Yeh, T.-H., Lu, C.-S., … Wang, H.-L. (2014). (G2019S) LRRK2 causes early-phase dysfunction of SNpc dopaminergic neurons and impairment of corticostriatal long-term depression in the PD transgenic mouse. *Neurobiology of Disease*, *68*, 190–199. http://doi.org/10.1016/j.nbd.2014.04.021

Chung, J. Y., Lee, J. W., Ryu, C. H., Min, H. K., Yoon, Y. J., Lim, M. J., & Park, C. H. (2015). 1-[2-(4-Benzyloxyphenoxy)Ethyl]Imidazole inhibits monoamine oxidase B and protects against neuronal loss and behavioral impairment in rodent models of Parkinson's disease. *Journal of Neuroscience Research*, *00*, n/a–n/a. http://doi.org/10.1002/jnr.23577

Conrady, C. D., Drevets, D. a., & Carr, D. J. J. (2010). Herpes simplex type I (HSV-1) infection of the nervous system: Is an immune response a good thing? *Journal of Neuroimmunology*, *220*(1-2), 1–9. http://doi.org/10.1016/j.jneuroim.2009.09.013

Cotman, C. W., Berchtold, N. C., & Christie, L. A. (2007). Exercise builds brain health: key roles of growth factor cascades and inflammation. *Trends in Neurosciences*. http://doi.org/10.1016/j.tins.2007.06.011

Crowley, S. D. (2014a). The cooperative roles of inflammation and oxidative stress in the pathogenesis of hypertension. *Antioxidants & Redox Signaling*, *20*(1), 102–120. http://doi.org/10.1089/ars.2013.5258

Crowley, S. D. (2014b). The cooperative roles of inflammation and oxidative stress in the pathogenesis of hypertension. *Antioxidants & Redox Signaling*, *20*(1), 102–120. http://doi.org/10.1089/ars.2013.5258

Daher, J. P. L., Abdelmotilib, H. A., Hu, X., Volpicelli-Daley, L. A., Moehle, M. S., Fraser, K. B., … West, A. B. (2015). Leucine-rich Repeat Kinase 2 (LRRK2) Pharmacological Inhibition Abates α-Synuclein Gene-induced Neurodegeneration. *Journal of Biological Chemistry*, *290*(32), 19433–19444. http://doi.org/10.1074/jbc.M115.660001

De Lau, L. M. L., & Breteler, M. M. B. (2006). Epidemiology of Parkinson's disease. *The Lancet. Neurology*, *5*(June), 525–535. http://doi.org/10.1016/S1474-4422(06)70471-9

Denning, T. L., Wang, Y., Patel, S. R., Williams, I. R., & Pulendran, B. (2007). Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. *Nature Immunology*, *8*(10), 1086–1094. http://doi.org/10.1038/ni1511

Dheen, S. T., Kaur, C., & Ling, E.-A. (2007). Microglial activation and its implications in the brain diseases. *Current Medicinal Chemistry*, *14*(11), 1189–1197. http://doi.org/10.2174/092986707780597961

Dinarello, C. a. (2000). Impact of basic research on tomorrow ' s medicine Proinflammatory Cytokines. *CHEST Journal*, *118*(2), 503–508. http://doi.org/10.1378/chest.117.4.1162

Dobrakowski, P. P., Machowska-Majchrzak, A. K., Labuz-Roszak, B., Majchrzak, K. G., & Kluczewska, E. (2014). MR-Guided Focused Ultrasound: A New Generation Treatment of Parkinsons Disease,

Essential Tremor and Neuropathic Pain. *Interventional Neuroradiology*, *20*, 0. http://doi.org/10.15274/INR-2014-10033

Drath, D. B., & Karnovsky, M. L. (1975). Superoxide production by phagocytic leukocytes. *The Journal of Experimental Medicine*, *141*(1), 257–262. http://doi.org/10.1084/jem.141.1.257

Dujardin, K., Langlois, C., Plomhause, L., Carette, A.-S., Delliaux, M., Duhamel, A., & Defebvre, L. (2014). Apathy in untreated early-stage Parkinson disease: Relationship with other non-motor symptoms. *Movement Disorders*, *29*(14), 1796–1801. http://doi.org/10.1002/mds.26058

Dupaul-Chicoine, J., Dagenais, M., & Saleh, M. (2013). Crosstalk between the intestinal microbiota and the innate immune system in intestinal homeostasis and inflammatory bowel disease. *Inflammatory Bowel Diseases*, *19*(10), 2227–37. http://doi.org/10.1097/MIB.0b013e31828dcac7

Finkel, T., & Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature*, *408*(6809), 239–247. http://doi.org/10.1038/35041687

Flood, P. M., Qian, L., Peterson, L. J., Zhang, F., Shi, J.-S., Gao, H.-M., & Hong, J.-S. (2011). Transcriptional Factor NF-κB as a Target for Therapy in Parkinson's Disease. *Parkinson's Disease*, *2011*, 216298. http://doi.org/10.4061/2011/216298

G. M. Petzinger, B. E. Fisher, S. McEwen, J. A. B. J. P. W. and M. W., & Jakowec. (2013). Cognitive Circuitry in Parkinson ' s Disease, *12*(7), 716–726. http://doi.org/10.1016/S1474-4422(13)70123-6.Exercise-enhanced

Gabay, C. (2006). Interleukin-6 and chronic inflammation. *Arthritis Research & Therapy*, *8 Suppl 2*, S3. http://doi.org/10.1186/ar1917

Giesert, F., Hofmann, A., Bürger, A., Zerle, J., Kloos, K., Hafen, U., … Wurst, W. (2013). Expression Analysis of Lrrk1, Lrrk2 and Lrrk2 Splice Variants in Mice. *PLoS ONE*, *8*(5), e63778. http://doi.org/10.1371/journal.pone.0063778

Gillardon, F., Schmid, R., & Draheim, H. (2012). Parkinson's disease-linked leucine-rich repeat kinase 2(R1441G) mutation increases proinflammatory cytokine release from activated primary microglial cells and resultant neurotoxicity. *Neuroscience*, *208*, 41–48. http://doi.org/10.1016/j.neuroscience.2012.02.001

Gómez-Suaga, P., Fdez, E., Blanca Ramírez, M., & Hilfiker, S. (2012). A link between autophagy and the pathophysiology of LRRK2 in Parkinson's disease. *Parkinson's Disease*. http://doi.org/10.1155/2012/324521

Goodwin, V. a., Richards, S. H., Taylor, R. S., Taylor, A. H., & Campbell, J. L. (2008). The effectiveness of exercise interventions for people with Parkinson's disease: A systematic review and meta-analysis. *Movement Disorders*, *23*(5), 631–640. http://doi.org/10.1002/mds.21922

Greggio, E., Civiero, L., Bisaglia, M., & Bubacco, L. (2012). Parkinson's disease and immune system: is the culprit LRRKing in the periphery? *Journal of Neuroinflammation*. http://doi.org/10.1186/1742-2094-9-94

Halliday, G. M., & McCann, H. (2010). The progression of pathology in Parkinson's disease. *Annals of the New York Academy of Sciences*. http://doi.org/10.1111/j.1749-6632.2009.05118.x

Heckman, M. G., Elbaz, A., Soto-Ortolaza, A. I., Serie, D. J., Aasly, J. O., Annesi, G., … Ross, O. A. (2014). The protective effect of LRRK2 p.R1398H on risk of Parkinson's disease is independent of MAPT and SNCA variants. *Neurobiology of Aging*, *35*(1), 266.e5–266.e14. http://doi.org/10.1016/j.neurobiolaging.2013.07.013

Herzig, M. C., Kolly, C., Persohn, E., Theil, D., Schweizer, T., Hafner, T., … Shimshek, D. R. (2011). LRRK2 protein levels are determined by kinase function and are crucial for kidney and lung homeostasis in mice. *Human Molecular Genetics*, *20*(21), 4209–4223. http://doi.org/10.1093/hmg/ddr348

Hickey, W. F. (1991). Migration of hematogenous cells through the blood-brain barrier and the initiation of CNS inflammation. *Brain Pathology (Zurich, Switzerland)*, *1*(2), 97–105. http://doi.org/10.1111/j.1750-3639.1991.tb00646.x

Hoefkens, E., Nys, K., John, J. M., Van Steen, K., Arijs, I., Van Der Goten, J., … Cleynen, I. (2013). Genetic association and functional role of Crohn disease risk alleles involved in microbial sensing, autophagy, and endoplasmic reticulum (ER) stress. *Autophagy*, *9*(12), 2046–2055. http://doi.org/10.4161/auto.26337

Holness, C. L., & Simmons, D. L. (1993). Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. *Blood*, *81*(6), 1607–1613. http://doi.org/7680921

Hou, G., Tian, R., Li, J., & Yuan, T.-F. (2014). Chronic Stress and Parkinson's Disease. *CNS Neuroscience & Therapeutics*, *20*(1), 1–2. http://doi.org/10.1111/cns.12209

Hughes, L. E., Altena, E., Barker, R. a., & Rowe, J. B. (2013). Perseveration and choice in parkinson's disease: The impact of progressive frontostriatal dysfunction on action decisions. *Cerebral Cortex*, *23*(7), 1572–1581. http://doi.org/10.1093/cercor/bhs144

Hunter, R. L., Cheng, B., Choi, D.-Y., Liu, M., Liu, S., Cass, W. A., & Bing, G. (2009). Intrastriatal lipopolysaccharide injection induces parkinsonism in C57/B6 mice. *Journal of Neuroscience Research*, *87*(8), 1913–1921. http://doi.org/10.1002/jnr.22012

Hybertson, B. M., Gao, B., Bose, S. K., & McCord, J. M. (2011). Oxidative stress in health and disease: The therapeutic potential of Nrf2 activation. *Molecular Aspects of Medicine*, *32*(4-6), 234–246. http://doi.org/10.1016/j.mam.2011.10.006

Ivanov, I. I., Atarashi, K., Manel, N., Brodie, E. L., Shima, T., Karaoz, U., … Littman, D. R. (2009). Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria. *Cell*, *139*(3), 485–498. http://doi.org/10.1016/j.cell.2009.09.033

Iwasaki, A., & Medzhitov, R. (2010). Regulation of adaptive immunity by the innate immune system. *Science (New York, N.Y.)*, *327*(5963), 291–295. http://doi.org/10.1126/science.1183021

Jenner, P., & Olanow, C. W. (1996). Oxidative stress and the pathogenesis of Parkinson ' s disease Oxidative stress and the pathogenesis of Parkinson ' s disease, (C). http://doi.org/10.1212/WNL.47.6

Kaltschmidt, B., & Kaltschmidt, C. (2009). NF-kappaB in the nervous system. *Cold Spring Harbor Perspectives in Biology*. http://doi.org/10.1101/cshperspect.a001271

Kamada, N., Kim, Y.-G., Sham, H. P., Vallance, B. A., Puente, J. L., Martens, E. C., & Nunez, G. (2012). Regulated Virulence Controls the Ability of a Pathogen to Compete with the Gut Microbiota. *Science*. http://doi.org/10.1126/science.1222195

Kavanagh, M. E., Doddareddy, M. R., & Kassiou, M. (2013). The development of CNS-active LRRK2 inhibitors using property-directed optimisation. *Bioorganic and Medicinal Chemistry Letters*. http://doi.org/10.1016/j.bmcl.2013.04.086

Khang, R., Park, C., & Shin, J.-H. (2015). Dysregulation of parkin in the substantia nigra of db/db and high-fat diet mice. *Neuroscience*, *294*, 182–192. http://doi.org/10.1016/j.neuroscience.2015.03.017

Kim, W. G., Mohney, R. P., Wilson, B., Jeohn, G. H., Liu, B., & Hong, J. S. (2000). Regional difference in susceptibility to lipopolysaccharide-induced neurotoxicity in the rat brain: role of microglia. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *20*(16), 6309–6316. http://doi.org/20/16/6309 [pii]

Kloss, C. U., Bohatschek, M., Kreutzberg, G. W., & Raivich, G. (2001). Effect of lipopolysaccharide on the morphology and integrin immunoreactivity of ramified microglia in the mouse brain and in cell culture. *Experimental Neurology*, *168*(1), 32–46. http://doi.org/10.1006/exnr.2000.7575

Konsman, J. P., Parnet, P., & Dantzer, R. (2002). Cytokine-induced sickness behaviour: Mechanisms and implications. *Trends in Neurosciences*, *25*(3), 154–159. http://doi.org/10.1016/S0166-2236(00)02088-9

Kreutzberg, G. W. (1996). Microglia: A sensor for pathological events in the CNS. *Trends in Neurosciences*. http://doi.org/10.1016/0166-2236(96)10049-7

Kumar, H., Kawai, T., & Akira, S. (2011). Pathogen recognition by the innate immune system. *International Reviews of Immunology*, *30*(1), 16–34. http://doi.org/10.3109/08830185.2010.529976

Landrigan, P. J., Sonawane, B., Butler, R. N., Trasande, L., Callan, R., & Droller, D. (2005). Early environmental origins of neurodegenerative disease in later life. *Environmental Health Perspectives*. http://doi.org/10.1289/ehp.7571

Lawrence, T. (2009). The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harbor Perspectives in Biology*. http://doi.org/10.1101/cshperspect.a001651

Lawson, L. J., Perry, V. H., Dri, P., & Gordon, S. (1990). Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. *Neuroscience*, *39*(1), 151–170. http://doi.org/10.1016/0306-4522(90)90229-W

Lee, J. Der, Huang, C. H., Yang, S. T., Chu, Y. H., Shieh, Y. Y., Chen, J. W., & Lin, K. J. (2013). MRI/SPECT-based diagnosis and CT-guided high-intensity focused-ultrasound treatment system in MPTP mouse model of Parkinson's disease. *Medical Engineering and Physics*, *35*(2), 222–230. http://doi.org/10.1016/j.medengphy.2012.01.006

Lee, J.-W., Tapias, V., Di Maio, R., Greenamyre, J. T., & Cannon, J. R. (2015a). Behavioral, neurochemical, and pathologic alterations in bacterial artificial chromosome transgenic G2019S leucine-rich repeated kinase 2 rats. *Neurobiology of Aging*, *36*(1), 505–518. http://doi.org/10.1016/j.neurobiolaging.2014.07.011

Lee, J.-W., Tapias, V., Di Maio, R., Greenamyre, J. T., & Cannon, J. R. (2015b). Behavioral, neurochemical, and pathologic alterations in bacterial artificial chromosome transgenic G2019S leucine-rich repeated kinase 2 rats. *Neurobiology of Aging*, *36*(1), 505–518. http://doi.org/10.1016/j.neurobiolaging.2014.07.011

Leisman, G., Braun-Benjamin, O., & Melillo, R. (2014). Cognitive-motor interactions of the basal ganglia in development. *Frontiers in Systems Neuroscience*, *8*(February), 16. http://doi.org/10.3389/fnsys.2014.00016

Li, G., Sun, S., Cao, X., Zhong, J., & Tong, E. (2004). LPS-induced degeneration of dopaminergic neurons of substantia nigra in rats. *Journal of Huazhong University of Science and Technology. Medical Sciences = Hua Zhong Ke Ji Da Xue Xue Bao. Yi Xue Ying De Wen Ban = Huazhong Keji Daxue Xuebao. Yixue Yingdewen Ban*, *24*(1), 83–86. http://doi.org/10.1007/BF02830714

Li, J.-Q., Tan, L., & Yu, J.-T. (2014a). The role of the LRRK2 gene in Parkinsonism. *Molecular Neurodegeneration*, *9*(1), 47. http://doi.org/10.1186/1750-1326-9-47

Li, J.-Q., Tan, L., & Yu, J.-T. (2014b). The role of the LRRK2 gene in Parkinsonism. *Molecular Neurodegeneration*, *9*(1), 47. http://doi.org/10.1186/1750-1326-9-47

Li, N., Ragheb, K., Lawler, G., Sturgis, J., Rajwa, B., Melendez, J. A., & Robinson, J. P. (2003). Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. *Journal of Biological Chemistry*, *278*(10), 8516–8525. http://doi.org/10.1074/jbc.M210432200

Lin, C.-H., Lin, J.-W., Liu, Y.-C., Chang, C.-H., & Wu, R.-M. (2014). Risk of Parkinson's disease following severe constipation: A nationwide population-based cohort study. *Parkinsonism & Related Disorders*, *20*(12), 1371–1375. http://doi.org/10.1016/j.parkreldis.2014.09.026

Litteljohn, D., Cummings, A., Brennan, A., Gill, A., Chunduri, S., Anisman, H., & Hayley, S. (2010). Interferon-gamma deficiency modifies the effects of a chronic stressor in mice: Implications for psychological pathology. *Brain, Behavior, and Immunity*, *24*(3), 462–473. http://doi.org/10.1016/j.bbi.2009.12.001

Litteljohn, D., Mangano, E., Clarke, M., Bobyn, J., Moloney, K., & Hayley, S. (2010). Inflammatory mechanisms of neurodegeneration in toxin-based models of Parkinson's disease. *Parkinson's Disease*, *2011*, 713517. http://doi.org/10.4061/2011/713517

Litteljohn, D., Nelson, E., & Hayley, S. (2014). IFN-Î³ differentially modulates memory-related processes under basal and chronic stressor conditions. *Frontiers in Cellular Neuroscience*, *8*. http://doi.org/10.3389/fncel.2014.00391

Liu, H., Ho, P., Tse, Z., Kung, M., Zhou, Z., Ramsden, D., & Ho, S. (2014). Leucine-Rich Repeat Kinase-2 (LRRK2) R1441G Knockin Mice Are More Susceptible To Rotenone Toxicity. *Movement Disorders*, *29*.

Lowder, T., Padgett, D. a., & Woods, J. a. (2005). Moderate exercise protects mice from death due to influenza virus. *Brain, Behavior, and Immunity*, *19*(5), 377–380. http://doi.org/10.1016/j.bbi.2005.04.002

Majno, G., & Joris, I. (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. *The American Journal of Pathology*, *146*(1), 3–15. http://doi.org/7856735

Mangano, E. N., & Hayley, S. (2009). Inflammatory priming of the substantia nigra influences the impact of later paraquat exposure: Neuroimmune sensitization of neurodegeneration. *Neurobiology of Aging*, *30*(9), 1361–1378. http://doi.org/10.1016/j.neurobiolaging.2007.11.020

Manzoni, C., Mamais, A., Dihanich, S., Abeti, R., Soutar, M. P. M., Plun-Favreau, H., … Lewis, P. A. (2013). Inhibition of LRRK2 kinase activity stimulates macroautophagy. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1833*(12), 2900–2910. http://doi.org/10.1016/j.bbamcr.2013.07.020

Marsh, L. (2013). Depression and Parkinson's disease: current knowledge. *Current Neurology and Neuroscience Reports*, *13*(12), 409. http://doi.org/10.1007/s11910-013-0409-5

Martyn, C. N. (1997). Infection in childhood and neurological diseases in adult life. *British Medical Bulletin*, *53*(1), 24–39.

Massano, J., & Garrett, C. (2012). Deep brain stimulation and cognitive decline in Parkinson's disease: A clinical review. *Frontiers in Neurology*, *APR*(April), 1–13. http://doi.org/10.3389/fneur.2012.00066

McGeer, P. L., & McGeer, E. G. (2004). Inflammation and neurodegeneration in Parkinson's disease. *Parkinsonism and Related Disorders*, *10*(SUPPL. 1), 3–7. http://doi.org/10.1016/j.parkreldis.2004.01.005

McGeer, P. L., Schwab, C., Parent, A., & Doudet, D. (2003). Presence of Reactive Microglia in Monkey Substantia Nigra Years after 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Administration. *Annals of Neurology*, *54*(5), 599–604. http://doi.org/10.1002/ana.10728

McNeely, M. E., Duncan, R. P., & Earhart, G. M. (2015). A comparison of dance interventions in people with Parkinson disease and older adults. *Maturitas*, 1–7. http://doi.org/10.1016/j.maturitas.2015.02.007

Mills, C. D. (2012). M1 and M2 Macrophages: Oracles of Health and Disease. *Critical Reviews in Immunology*, *32*(6), 463–88. http://doi.org/03b91cfa635b86ee,7acd81f604dc8b0c [pii]

Miyazaki, I., & Asanuma, M. (2008). Dopaminergic neuron-specific oxidative stress caused by dopamine itself. *Acta Medica Okayama*, *62*(3).

Mo, C., Hannan, A. J., & Renoir, T. (2015). Environmental factors as modulators of neurodegeneration: Insights from gene–environment interactions in Huntington's disease. *Neuroscience & Biobehavioral Reviews*, *52*, 178–192. http://doi.org/10.1016/j.neubiorev.2015.03.003

Mollenhauer, B., Trautmann, E., Sixel-Döring, F., Wicke, T., Ebentheuer, J., Schaumburg, M., … Trenkwalder, C. (2013). Nonmotor and diagnostic findings in subjects with de novo Parkinson disease of the DeNoPa cohort. *Neurology*, *81*(14), 1226–1234. http://doi.org/10.1212/WNL.0b013e3182a6cbd5

Mount, M. P., Lira, A., Grimes, D., Smith, P. D., Faucher, S., Slack, R., … Park, D. S. (2007). Involvement of interferon-gamma in microglial-mediated loss of dopaminergic neurons. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *27*(12), 3328–3337. http://doi.org/10.1523/JNEUROSCI.5321-06.2007

Murai, M., Turovskaya, O., Kim, G., Madan, R., Karp, C. L., Cheroutre, H., & Kronenberg, M. (2009). Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nature Immunology*, *10*(11), 1178–1184. http://doi.org/10.1038/ni.1791

Nagatsu, T., & Sawada, M. (2005). Inflammatory process in Parkinson's disease: role for cytokines. *Current Pharmaceutical Design*, *11*(8), 999–1016. http://doi.org/10.2174/1381612053381620

Nemenova, N. M., Turbina, N. S., Novikova, T. S., & Faĭnshte in, F. E. (1976). Several clinic-morphologic aspects and problems concerning the pathogenesis of the Marchiafava-Miche li syndrome (paroxysmal nocturnal hemoglobinuria. *Arkhiv Patologii*, *38*(1), 52–58.

Nenci, A., Becker, C., Wullaert, A., Gareus, R., van Loo, G., Danese, S., … Pasparakis, M. (2007). Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature*, *446*(7135), 557–561. http://doi.org/10.1038/nature05698

Noval Rivas, M., Burton, O. T., Wise, P., Zhang, Y. Q., Hobson, S. A., Garcia Lloret, M., … Chatila, T. A. (2013). A microbiota signature associated with experimental food allergy promotes allergic

sensitization and anaphylaxis. *Journal of Allergy and Clinical Immunology*, *131*(1), 201–212. http://doi.org/10.1016/j.jaci.2012.10.026

Obeso, J. a., Rodriguez-Oroz, M. C., Stamelou, M., Bhatia, K. P., & Burn, D. J. (2014). The expanding universe of disorders of the basal ganglia. *The Lancet*, *384*(9942), 523–531. http://doi.org/10.1016/S0140-6736(13)62418-6

Olanow, C. W., Agid, Y., Mizuno, Y., Albanese, A., Bonuccelli, U., Damier, P., … Stocchi, F. (2004). Levodopa in the treatment of Parkinson's disease: current controversies. *Movement Disorders : Official Journal of the Movement Disorder Society*, *19*(9), 997–1005. http://doi.org/10.1002/mds.20243

Olanow, C. W., & Tatton, W. G. (1999). Etiology and Pathogenesis of Parkinson ' S Disease.

Ott, M., Gogvadze, V., Orrenius, S., & Zhivotovsky, B. (2007). Mitochondria, oxidative stress and cell death. *Apoptosis*, *12*(5), 913–922. http://doi.org/10.1007/s10495-007-0756-2

Pachter, J. S., de Vries, H. E., & Fabry, Z. (2003). The blood-brain barrier and its role in immune privilege in the central nervous system. *Journal of Neuropathology and Experimental Neurology*, *62*(6), 593–604.

Park, G., Park, Y. J., Yang, H. O., & Oh, M. S. (2013). Ropinirole protects against 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-induced neurotoxicity in mice via anti-apoptotic mechanism. *Pharmacology Biochemistry and Behavior*, *104*(1), 163–168. http://doi.org/10.1016/j.pbb.2013.01.017

Passos, E., Ascensão, A., Martins, M. J., & Magalhães, J. (2015). Endoplasmic reticulum stress response in non-alcoholic steatohepatitis: The possible role of physical exercise. *Metabolism*, 1–13. http://doi.org/10.1016/j.metabol.2015.02.003

PD MED Collaborative Group. (2014). Long-term effectiveness of dopamine agonists and monoamine oxidase B inhibitors compared with levodopa as initial treatment for Parkinson's disease (PD MED): a large, open-label, pragmatic randomised trial. *The Lancet*, *384*(9949), 1196–1205. http://doi.org/10.1016/S0140-6736(14)60683-8

Qureshi, S. U., Amspoker, A. B., Calleo, J. S., Kunik, M. E., & Marsh, L. (2012). Anxiety disorders, physical illnesses, and health care utilization in older male veterans with Parkinson disease and comorbid depression. *Journal of Geriatric Psychiatry and Neurology*, *25*(4), 233–9. http://doi.org/10.1177/0891988712466458

Reynolds, A., Doggett, E. A., Riddle, S. M., Lebakken, C. S., & Nichols, R. J. (2014). LRRK2 kinase activity and biology are not uniformly predicted by its autophosphorylation and cellular phosphorylation site status. *Frontiers in Molecular Neuroscience*, *7*. http://doi.org/10.3389/fnmol.2014.00054

Ridderinkhof, K. R., & Brass, M. (2015). Journal of Physiology - Paris How Kinesthetic Motor Imagery works : A predictive-processing theory of visualization in sports and motor expertise. *Journal of Physiology Paris*, (March). http://doi.org/10.1016/j.jphysparis.2015.02.003

Riederer, P., & Wuketich, S. (1976). Time course of nigrostriatal degeneration in parkinson's disease. A detailed study of influential factors in human brain amine analysis. *Journal of Neural Transmission (Vienna, Austria : 1996)*, *38*(3-4), 277–301. http://doi.org/10.1007/BF01249445

Rinne, U. K., Bracco, F., Chouza, C., Dupont, E., Gershanik, O., Marti Masso, J. F., … Marsden, C. D. (1998). Early treatment of Parkinson's disease with cabergoline delays the onset of motor complications. Results of a double-blind levodopa controlled trial. In *Drugs* (Vol. 55, pp. 23–30). http://doi.org/10.2165/00003495-199855001-00004

Rodriguez-Oroz, M. C., Obeso, J. a., Lang, a. E., Houeto, J. L., Pollak, P., Rehncrona, S., … Van Blercom, N. (2005). Bilateral deep brain stimulation in Parkinson's disease: A multicentre study with 4 years follow-up. *Brain*, *128*(10), 2240–2249. http://doi.org/10.1093/brain/awh571

Rogove, A. D., Lu, W., & Tsirka, S. E. (2002). Microglial activation and recruitment, but not proliferation, suffice to mediate neurodegeneration. *Cell Death and Differentiation*, *9*(8), 801–806. http://doi.org/10.1038/sj.cdd.4401041

Rylander, R. (2002). Endotoxin in the environment--exposure and effects. *Journal of Endotoxin Research*, *8*(4), 241–252. http://doi.org/10.1179/096805102125000452

Saha, S., Ash, P. E. A., Gowda, V., Liu, L., Shirihai, O., & Wolozin, B. (2015). Mutations in LRRK2 potentiate age-related impairment of autophagic flux. *Molecular Neurodegeneration*, *10*(1), 26. http://doi.org/10.1186/s13024-015-0022-y

Schapira, A. H., & Jenner, P. (2011). Etiology and pathogenesis of Parkinson's disease. *Movement Disorders*, *26*(6), 1049–1055. http://doi.org/10.1002/mds.23732

Scheperjans, F., Aho, V., Pereira, P. a. B., Koskinen, K., Paulin, L., Pekkonen, E., … Auvinen, P. (2014). Gut microbiota are related to Parkinson's disease and clinical phenotype. *Movement Disorders*, *30*(3), n/a–n/a. http://doi.org/10.1002/mds.26069

Shacter, E., & Weitzman, S. a. (2002). Chronic inflammation and cancer. *Oncology (Williston Park, N.Y.)*, *16*(2), 217–226, 229; discussion 230–232.

Sharma, N., & Nehru, B. (2015). Characterization of the lipopolysaccharide induced model of Parkinson's disease: Role of oxidative stress and neuroinflammation. *Neurochemistry International*, *87*, 92–105. http://doi.org/10.1016/j.neuint.2015.06.004

Skibinski, G., Nakamura, K., Cookson, M. R., & Finkbeiner, S. (2014). Mutant LRRK2 toxicity in neurons depends on LRRK2 levels and synuclein but not kinase activity or inclusion bodies. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*. http://doi.org/10.1523/JNEUROSCI.2712-13.2014

Skulina, C., Schmidt, S., Dornmair, K., Babbe, H., Roers, A., Rajewsky, K., … Goebels, N. (2004). Multiple sclerosis: Brain-infiltrating CD8+ T cells persist as clonal expansions in the cerebrospinal fluid and blood. *Proceedings of the National Academy of Sciences*, *101*(8), 2428–2433. http://doi.org/10.1073/pnas.0308689100

Smeding, H. M. M., Speelman, J. D., Huizenga, H. M., Schuurman, P. R., & Schmand, B. (2011). Predictors of cognitive and psychosocial outcome after STN DBS in Parkinson's Disease. *Journal of Neurology, Neurosurgery, and Psychiatry*, *82*(7), 754–760. http://doi.org/10.1136/jnnp.2007.140012

Stollg, G., & Jander, S. (1999). The role of microglia and macrophages in the pathophysiology of the CNS. *Progress in Neurobiology*, *58*(3), 233–247. http://doi.org/10.1016/S0301-0082(98)00083-5

Sugama, S., Yang, L., Cho, B. P., DeGiorgio, L. A., Lorenzl, S., Albers, D. S., … Joh, T. H. (2003). Age-related microglial activation in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurodegeneration in C57BL/6 mice. *Brain Research*, *964*(2), 288–294. http://doi.org/10.1016/S0006-8993(02)04085-4

Sulzer, D. (2007). Multiple hit hypotheses for dopamine neuron loss in Parkinson's disease. *Trends in Neurosciences*. http://doi.org/10.1016/j.tins.2007.03.009

Sung, V. W., Watts, R. L., Schrandt, C. J., Guthrie, S., Wang, D., Amara, A. W., … Harrison, C. (2014). Stimulation in Parkinson ' s Disease, *119*(6), 1530–1536. http://doi.org/10.3171/2013.8.JNS122025.Clinical

Takahashi, M., & Yamada, T. (1999). Viral etiology for Parkinson's disease--a possible role of influenza A virus infection. *Japanese Journal of Infectious Diseases*, *52*(3), 89–98.

Tanaka, S., Ishii, A., Ohtaki, H., Shioda, S., Yoshida, T., & Numazawa, S. (2013). Activation of microglia induces symptoms of Parkinson's disease in wild-type, but not in IL-1 knockout mice. *Journal of Neuroinflammation*, *10*(1), 143. http://doi.org/10.1186/1742-2094-10-143

Tanner, C. M., Ross, G. W., Jewell, S. A., Hauser, R. A., Jankovic, J., Factor, S. A., … Langston, J. W. (2009). Occupation and Risk of Parkinsonism. *Archives of Neurology*, *66*(9). http://doi.org/10.1001/archneurol.2009.195

Tatton, W. G., Warner, T. T., Schapira, A. H. V, Chalmers-Redman, R., Brown, D., & Tatton, N. (2003). Genetic and Environmental Factors in the Cause of Parkinson﷽s Disease. *Annals of Neurology*, *53 Suppl 3*, S61–70; discussion S70–2. http://doi.org/10.1002/ana.10489.Address

Teschke, K., Marion, S. A., Tsui, J. K. C., Shen, H., Rugbjerg, K., & Harris, M. A. (2014). Parkinson's Disease and Occupation: Differences in Associations by Case Identification Method Suggest Referral Bias. *American Journal of Industrial Medicine*, *57*(2), 163–171. http://doi.org/10.1002/ajim.22272

Tong, Y., Giaime, E., Yamaguchi, H., Ichimura, T., Liu, Y., Si, H., … Shen, J. (2012). Loss of leucine-rich repeat kinase 2 causes age-dependent bi-phasic alterations of the autophagy pathway. *Molecular Neurodegeneration*, *7*(1), 2. http://doi.org/10.1186/1750-1326-7-2

Tremblay, L., Worbe, Y., Thobois, S., Sgambato-Faure, V., & Féger, J. (2015). Selective dysfunction of basal ganglia subterritories: From movement to behavioral disorders. *Movement Disorders*, *00*(00), n/a–n/a. http://doi.org/10.1002/mds.26199

Turrens, J. F., Alexandre, a, & Lehninger, a L. (1985). Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Archives of Biochemistry and Biophysics*, *237*(2), 408–414. http://doi.org/10.1016/0003-9861(85)90293-0

Universitaria, C. (2001). Deep-brain stimulation of the subthalamic nucleus or the pars interna of the globus pallidus in Parkinson's disease. *The New England Journal of Medicine*, *345*(13), 956–963. http://doi.org/10.1056/NEJMoa000827

Vaziri, N. D., Dicus, M., Ho, N. D., Boroujerdi-Rad, L., & Sindhu, R. K. (2003). Oxidative stress and dysregulation of superoxide dismutase and NADPH oxidase in renal insufficiency. *Kidney International*, *63*(1), 179–185. http://doi.org/10.1046/j.1523-1755.2003.00702.x

Vercammen, E., Staal, J., & Beyaert, R. (2008). Sensing of viral infection and activation of innate immunity by toll-like receptor 3. *Clinical Microbiology Reviews*, *21*(1), 13–25. http://doi.org/10.1128/CMR.00022-07

Wadley, A. J., Veldhuijzen Van Zanten, J. J. C. S., & Aldred, S. (2013). The interactions of oxidative stress and inflammation with vascular dysfunction in ageing: The vascular health triad. *Age*, *35*(3), 705–718. http://doi.org/10.1007/s11357-012-9402-1

Wang, D., Xu, L., Lv, L., Su, L.-Y., Fan, Y., Zhang, D.-F., … Yao, Y.-G. (2014a). Association of the LRRK2 genetic polymorphisms with leprosy in Han Chinese from Southwest China. *Genes and Immunity*, *16*(2), 112–119. http://doi.org/10.1038/gene.2014.72

Wang, D., Xu, L., Lv, L., Su, L.-Y., Fan, Y., Zhang, D.-F., … Yao, Y.-G. (2014b). Association of the LRRK2 genetic polymorphisms with leprosy in Han Chinese from Southwest China. *Genes and Immunity*, *16*(2), 112–119. http://doi.org/10.1038/gene.2014.72

Wang, X., Lu, G., Hu, X., Tsang, K., Kwong, W., Wu, F., … Poon, W. (2012). Quantitative assessment of gait and neurochemical correlation in a classical murine model of Parkinson's disease. *BMC Neuroscience*, *13*(1), 142. http://doi.org/10.1186/1471-2202-13-142

West, A. B., Cowell, R. M., Daher, J. P. L., Moehle, M. S., Hinkle, K. M., Melrose, H. L., … Volpicelli-Daley, L. A. (2014). Differential LRRK2 expression in the cortex, striatum, and substantia nigra in transgenic and nontransgenic rodents. *Journal of Comparative Neurology*, *522*(11), 2465–2480. http://doi.org/10.1002/cne.23583

Williamson, J. S. P., Sykes, K. C., & Stohlman, S. A. (1991). Characterization of brain-infiltrating mononuclear cells during infection with mouse hepatitis virus strain JHM. *Journal of Neuroimmunology*, *32*(3), 199–207. http://doi.org/10.1016/0165-5728(91)90189-E

Wolburg, H., & Lippoldt, A. (2002). Tight junctions of the blood-brain barrier: Development, composition and regulation. *Vascular Pharmacology*. http://doi.org/10.1016/S1537-1891(02)00200-8

Wu, C., Orozco, C., Boyer, J., Leglise, M., Goodale, J., Batalov, S., … Su, A. I. (2009). BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources. *Genome Biology*, *10*(11), R130. http://doi.org/10.1186/gb-2009-10-11-r130

Wu, Y.-R., Chang, K.-H., Chang, W.-T., Hsiao, Y.-C., Hsu, H.-C., Jiang, P.-R., … Chen, C.-M. (2013). Genetic Variants ofLRRK2 in Taiwanese Parkinson's Disease. *PLoS ONE*, *8*(12), e82001. http://doi.org/10.1371/journal.pone.0082001

Xu, H., Barnes, G. T., Yang, Q., Tan, G., Yang, D., Chou, C. J., … Others. (2003). Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *Journal of Clinical Investigation*, *112*(12), 1821–1830. http://doi.org/10.1172/JCI200319451.Introduction

Xu, H., Liew, L. N., Kuo, I. C., Huang, C. H., Goh, D. L. M., & Chua, K. Y. (2008). The modulatory effects of lipopolysaccharide-stimulated B cells on differential T-cell polarization. *Immunology*, *125*(2), 218–228. http://doi.org/10.1111/j.1365-2567.2008.02832.x

Xue, P., Zheng, M., Gong, P., Lin, C., Zhou, J., Li, Y., … Hu, Y. (2015). Single Administration of Ultra-Low-Dose Lipopolysaccharide in Rat Early Pregnancy Induces TLR4 Activation in the Placenta Contributing to Preeclampsia. *PLOS ONE*, *10*(4), e0124001. http://doi.org/10.1371/journal.pone.0124001

Zou. (2015). Do physical exercise and reading reduce the risk of Parkinson ' s disease ? a cross-sectional study on factors associated with Parkinson ' s disease in elderly Chinese veterans, 695–700.