SEX AND LRRK2 GENOTYPE DIFFERENCES IN PD

Sex and LRRK2 Genotype Differences in Inflammation and Dopaminergic Neurodegeneration in a
Multi-Hit Model of Parkinson's Disease

by

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Abstract

Parkinson's disease (PD) is a multifactorial condition arising from a constellation of environmental insults in conjunction with genetic vulnerabilities, sex differences, and aging. The objective of the current thesis was to explicate potential additive/synergistic effects of genetic LRRK2 anomalies and environmental risk factors as they pertain to motor behaviour, nigrostriatal dopaminergic degeneration, and indices of neuroinflammation. Moreover, since the male sex represents a significant risk factor in PD, sexual dimorphisms were addressed. To achieve this, male and female mice bearing the G2019S-LRRK2 mutation were exposed to a systemic lipopolysaccharide (LPS) + paraquat (PQ) neurotoxicant challenge. Contrary to expectations, no additive/synergistic effects were observed between the G2019S mutation and LPS+PQ exposure. In fact, male G2019S mice appeared to be protected from the degenerative effects of LPS+PQ at this age. Moreover, an unanticipated sex by treatment interaction was found, such that female mice displayed greater deficits in gait and greater loss in nigral TH-positive neurons compared to males.

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List of Abbreviations

6-OHDA = 6-hydroxydopamine
ANOVA = analysis of variance
ATP = adenosine triphosphate
BAC = bacterial artificial chromosome
BBB = blood brain barrier
Ca⁺⁺ = Calcium
CD14 = cluster of differentiation 14
CD68 = cluster of differentiation 68
CNS = central nervous system
COR = carboxyl-terminal of Roc
DA = dopamine
DAT = dopamine transporter
DHT = dihydrotestosterone
ENS = enteric nervous system
ERK = extracellular signal-regulated kinases
ETC = electron transport chain
GSH = glutathione
GSK3 β = glycogen synthase 3 beta
GTP = guanosine triphosphate
IFN- γ = interferon-gamma
IL-1 β = interleukin-1 beta
iNOS = inducible nitric oxide synthase
JNK = c-Jun-N-terminal kinase
LB = Lewy body
LN = Lewy neurite
LPS = lipopolysaccharide
LRRK2 = leucine-rich repeat kinase 2
MAPK = mitogen-activated protein kinase
MKK4 = mitogen-activated protein kinase kinase 4
MLK = mixed lineage kinase
MOA = monoamine oxidase
MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP⁺ = 1-methyl-4-phenylpyridinium
MD2 = myeloid differentiation protein 2
NADPH = nicotinamide adenine dinucleotide phosphate
NF- κ B = nuclear factor-kappa B
NGS = normal goat serum
PB = phosphate buffer
PBS = phosphate buffer saline
PD = Parkinson's disease
PFA = paraformaldehyde
PINK1 = PTEN-induced putative kinase 1
PQ = paraquat
PRR = pattern recognition receptors
ROC = ras of complex
ROS = reactive oxygen species

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SN = substantia nigra

SNc = substantia nigra pars compacta

SRY = sex-determining region Y

TLR = toll-like receptor

TLR4 = toll-like receptor 4

TNF- α = tumor necrosis factor-alpha

TH = tyrosine hydroxylase

UCP = uncoupling protein

VMAT2 = vesicular monoamine transporter 2

WT = wild-type

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Sex and LRRK2 genotype differences in inflammation and dopaminergic neurodegeneration in a multi-hit model of Parkinson's disease

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, affecting approximately 0.3% of the population worldwide. This rises rapidly to 3% for individuals over the age of 65, demonstrating the age-related progressive nature of the disease (Dexter & Jenner, 2013). Biologically, PD is characterized primarily by the degeneration of dopamine (DA) containing neurons in the substantia nigra pars compacta (SNc) resulting in reduced release of DA from the downstream terminals in the striatum (Langston, 2006). Additionally, PD neuropathology is also identified by the intracellular formation of alpha-synuclein containing Lewy body (LB) and Lewy neurite (LN) inclusions (Duda et al., 2000). LB and LN inclusions have been shown to first appear in the peripheral gut wall of the enteric nervous system (ENS) and in the olfactory bulbs of the central nervous system (CNS) (Braak et al., 2003a, Wakabayashi et al., 1993; Wakabayashi, 1990). From the ENS, inclusions travel retrograde through the dorsal motor nucleus of the vagus nerve to the nuclei of the lower brainstem, which eventually expands throughout the nigrostriatal pathway of the mid- and forebrain (Braak et al., 2005). Alternatively, inclusions also travel anterograde from the olfactory bulbs to the fore- and midbrain (Braak et al., 2003a). Since these toxic inclusions preferably form within neurons containing long, unmyelinated axons, the nigrostriatal neurons are particularly susceptible (Braak et al., 2004). As the disease progresses further, LB and LN inclusions expand throughout the brain, eventually reaching the cerebral cortex (Braak et al., 2003). Appropriately, this distinct pathological progression of neural damage is closely linked to the onset and severity of specific clinical symptoms.

Among the neuropathological changes that occur in PD, there is little doubt that the degeneration of dopamine neurons in the nigrostriatal pathway is responsible for the characteristic motor deficits (Sulzer & Surmeier, 2013). These motor deficits include resting tremors, muscle rigidity, bradykinesia (i.e. slowness of movement), gait (i.e. pattern of movement of the limbs) dysfunction, and

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postural instability (Jancovic, 2008). Cardinal symptoms only become apparent after an approximated 30-50% loss of DA cells from the SNc and a 50-80% loss of DA from the striatum has occurred (Wu et al., 2011). Moreover, the nigrostriatal degeneration is estimated to manifest itself for up to six years prior to symptom onset and clinical diagnosis (Marek et al., 2001). Furthermore, the appearance of these clinical motor deficits is associated with the formation of LB and LN inclusions in the remaining DA neurons of the nigrostriatal pathway (Braak et al., 2003a). Similar to the antecedent neurodegeneration, the nigrostriatal inclusions emerge years after their first appearance in the ENS and olfactory bulbs (Braak et al., 2003a).

There appears to be a distinct prodromal period characterized by advancing degeneration of the DAergic nigrostriatal pathway and progressive expansion of LB and LN inclusions prior to the onset of clinical motor deficits. Interestingly, this prodromal period is increasingly identified by the expression of non-motor symptoms. Indeed, autonomic dysfunctions such as gastrointestinal disruptions (Abbott et al., 2001) and olfactory abnormalities (Ponsen et al., 2004; Ponsen et al., 2009) appear during this time. It is argued that these symptoms emerge as a consequence of the Lewy inclusion formation within the ENS and olfactory bulbs, respectively. Additional non-motor deficits, including neurobehavioural abnormalities such as anxiety and depression, as well as cognitive deficits, such as an increased risk for dementia, have also been documented. The appearance of these symptoms has been shown to appear both prior to clinical diagnosis (i.e. symptoms of depression and anxiety) and later on in the disease as the neuropathology expands towards the cerebral cortex (i.e. deficits in cognition). Thus, while PD is fundamentally defined by the damage that occurs to the nigrostriatal pathway, the presence of these diverse, non-motor symptoms are evidence for a more widespread degenerative and disruptive process.

As advances are made in the study of PD, it is becomingly increasingly clear that the etiopathogenesis of this disease is complex. To date, the pathogenic mechanisms involved in the degenerative onset and progression of PD remain to be fully understood. That being said, accumulating

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evidence consistently implicates oxidative stress and neuroinflammatory mechanisms as front-runners in explaining the degenerative process. While the etiology of PD is considered unknown, it is generally accepted that PD is a multifactorial condition arising from a constellation of environmental risk factors, in conjunction with genetic vulnerabilities, sex differences, and aging (Betabart et al., 2002; Keppa et al., 2006). It is presumed that interaction between these risk factors precipitates neuroinflammatory and oxidative stress processes that together give rise to the PD pathology. Currently, research in PD has moved towards better understanding the multifactorial aspect of the disease.

Oxidative Stress and PD Pathology

The term oxidative stress refers to a disturbance in the cellular pro-oxidant-antioxidant balance in the favour of the former. Pro-oxidants are chemicals that promote the synthesis of reactive oxygen species (ROS), while inhibiting antioxidant systems from detoxifying the reactive intermediates (Dias et al., 2013). ROS are generated from the activation of molecular oxygen, which can occur directly through several pathways, including reactions with reduced metals and toxins, or indirectly via the activation of certain enzymes, such as monoamine oxidase (MAO) and NADPH oxidases (Dias et al., 2013). Examples of ROS include, peroxides and superoxides, which under homeostatic conditions play an important role in cell signaling (Fomenko et al., 2011). However, when produced in excess during oxidative stress, these chemically reactive molecules can have detrimental impacts on cellular structures by modifying the nucleic acids, proteins, lipids, and carbohydrates of the cell. These modifications in turn alter a wide range of intracellular signaling pathways. In particular, oxidative stress has been widely shown to facilitate apoptotic processes (Filomeni & Ciriolo, 2006; Ryter et al., 2007; West & Marnett, 2006). As such, in the brain, these unfavourable regulations can disrupt neuronal signaling and even result in neuronal death. Accordingly, oxidative stress has been implicated as a critical process underlying the pathology associated with a number of neurological conditions.

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Over the years, accumulating evidence has targeted oxidative stress as the mechanism underlying the cascade in events that lead to cellular dysfunction and loss in PD (Jenner & Olanow, 2006; Schapira & Jenner, 2011). Indeed, postmortem brain analyses have shown that the substantia nigra (SN) of PD patients contains higher levels of oxidized lipids (Bosco et al., 2006), proteins, and DNA (Nakabeppu et al., 2007) compared to age matched controls. Accompanying this are reduced nigrostriatal levels in the antioxidant, reduced glutathione (GSH) (Zeevalk et al., 2008). Interestingly, it has been shown that DA containing neurons are especially vulnerable to ROS damage due to the highly active redox state of the neurotransmitter DA itself (Hwang et al., 2013; Zigmond et al., 2002). Under homeostatic conditions the majority of DA is taken up and stored in vesicles where it is protected from unfavourable interactions. However, when this process is disrupted, excess DA accumulates within the cytosol where it can be spontaneously or enzymatically oxidized into DA quinone reactive species (Hwang et al., 2013). These compounds in turn, promote cytotoxicity by modifying protein structure and function. Notably, DA quinone has been shown to covalently modify a number of proteins whose dysfunction are linked to PD pathology, such as alpha-synuclein (Martinez-Vicente, 2008) and parkin (LaVoie et al., 2005). As expected, this degree of oxidative damage directly correlates with cytosolic DA levels. For instance, if the vesicular storage of DA is inhibited by blocking vesicular monoamine transporter 2 (VMAT2), the consequential rise in cytosolic DA renders DA neurons more susceptible to toxin-induced oxidative stress (Uhl et al., 2000). Similarly, if DA reuptake by the nigrostriatal terminals is enhanced by increased dopamine transporter (DAT) expression, ROS production within these neurons is augmented (Dias et al., 2013). Taken together, DAergic neurons appear to be intrinsically more prone to generate ROS due to the highly reactive redox state of DA. Consequently, any event that triggers further oxidative stress can be harmful to these already vulnerable cells.

Substantial evidence supports the involvement of mitochondrial dysfunction in the production of ROS within DA neurons. Mitochondria are the powerhouse organelles of the cell that function

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primarily in extracting energy in the form of ATP from metabolic fuels such as glucose. This is achieved via the transport of electrons through the protein complexes of the electron transport chain (ETC) by redox reactions. Damage to the complexes of the ETC can cause a leakage of electrons, which consequently leads to ROS generation. Interestingly, post mortem analyses have revealed that PD patients have higher numbers of ETC deficient DA neurons (Bender et al., 2006), with particularly reduced activity in complex I (Parker et al., 1989). Moreover, it has been shown that systemic treatment with complex I inhibitors, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), results in the preferential cytotoxicity of DAergic neurons (Betarbet et al., 2002). As such, external toxins like MPTP have been used throughout the years to model nigrostriatal DAergic degeneration. Together these data demonstrate that the dysregulation of mitochondrial activity provides another significant source of oxidative stress that is associated with PD pathology. Not all toxins, however, exert their cytotoxic effects by inhibiting ETC activity. Indeed, exposure to herbicides, such as paraquat, result in nigrostriatal degeneration via oxidative stress mechanisms that are independent of mitochondrial functioning. Thus, providing yet another means for which oxidative stress results in DAergic cytotoxicity in PD.

Paraquat: An oxidative stressor implicated in PD. Paraquat (PQ), 1,1'-dimethyl 4,4'-bipyridinium dichloride, is a highly toxic herbicide widely used to control weed growth in a variety of crops. In humans, the acute ingestion of PQ accidentally or for suicidal purposes causes multi-organ failure with particularly severe pulmonary lesions, which are often fatal. Over the past three decades, accumulating epidemiological data have implicated chronic PQ exposure and chronic herbicide exposure in general, as a risk factor for developing PD. In a study conducted in Taiwan between 1993 and 1995 that looked at 120 PD patients and 240 hospital controls, approximately 46 PD cases and 40 controls were exposed to herbicides (Liou et al., 1997). This corresponded to a relative risk ratio of 2.89, which rose to 6.72 when exposure to herbicides was greater than 20 years (Liou et al., 1997).

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Similarly, in a study conducted in the United States between 2004 and 2007 that examined 519 PD cases and 511 paired controls, herbicide exposure was associated with a two-fold increased risk for developing PD (Tanner et al., 2009). In response to this, the European Union banned the use of paraquat in 2007. However, many countries, including Canada have yet to do so. In fact, it has been reported that developing countries such as China and India have increased their use of herbicides (Bové & Perier, 2012). As such, much work has been dedicated towards understanding the potential neurobiological and behavioural deficits PQ is suspected to induce as it relates to PD (Bové & Perier, 2012). Moreover, because of this epidemiological data, PQ has become an intriguing alternative toxin model to that of the commonly used MPTP model of PD. Indeed, unlike MPTP, whose direct exposure is limited to drug users and chemists, PQ is more readily exposed to the general population as a herbicide, and thus holds greater ecological validity.

Since PQ is chemically similar to MPP⁺, the reactive metabolite of MPTP, it was hypothesized that PQ exhibits its toxic effects by similarly generating ROS via the inhibition of mitochondrial complex I. Contrary to expectations, it turned out that PQ is not an efficient inhibitor of mitochondrial complex I (Richardson et al., 2005), and thus exerts its toxic effects via an alternate mechanism. Indeed, it has since been shown that PQ induces its cytotoxic actions through a process of redox cycling. In its native divalent cation form for which it is ingested as, PQ is unable to be taken up by cells. PQ must first be reduced into its monovalent cation form via oxidoreductases such as, NADPH oxidases (Cristovao et al., 2009). In the brain the inhibition of DAT, protects against PQ-induced toxicity (McCormack & Di Monte, 2003) suggesting that DAT plays a role in the cellular uptake of reduced PQ. Once sequestered by DAergic neurons via DAT transport, reduced PQ is rapidly oxidized back into its divalent cation form resulting in ROS byproducts (McCormack et al., 2005; Bonneh-Barkay et al., 2005a; Bonneh-Barkay et al., 2005b). As this cyclic process persists ROS byproducts accumulate creating an oxidatively stressed environment in which reactive cytosolic DA can be readily oxidized. In

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deed, PQ toxicity is associated with an increase in DA oxidative metabolism within the SN, evidenced by increased levels in oxidized DA coupled with decreased levels in GSH (Kang et al., 2009). PQ has also been shown to enhance available cytosolic DA by potentiating alpha-synuclein induced toxicity (Norris et al., 2007). Specifically, PQ causes the aggregation of mutated alpha-synuclein within the SN (Goers et al., 2003; Manning-Bog et al., 2002), which, among many of its disruptions, induces a reduction in vesicle number and consequently an accumulation in cytoplasmic DA (Wood et al., 2006). Resultantly, more DA is available to be oxidized. Taken together, PQ exposure results in the accumulation of ROS in DAergic neurons via a process of redox cycling, providing yet another means for which oxidative stress can be induced in PD pathology.

Over the years, *in vivo* studies using rodents have enabled researchers to further assess the neurobiological and behavioural consequences of systemic PQ exposure. Not surprisingly, due to the high polarity of native PQ with its two positive charges, PQ does not readily cross the lipophilic blood brain barrier (BBB). As such, PQ is thought to reach the brain via a neutral amino acid transporter (Shimizu et al., 2001), via associated structures outside the brain (i.e. the pineal gland and cerebral ventricles), or via leaky regions in the BBB (i.e. olfactory bulb, hypothalamus and the area of postrema) (Naylor et al., 1995). Regardless of its mechanism of entry into the CNS, systemic PQ exposure has been shown to provoke a dose-dependent and length of exposure dependent loss of DA neurons in the SNc (Brooks et al., 1999; Litteljohn et al., 2010; McCormack et al., 2002; Purisai et al., 2007). As expected, this loss in nigral DA neurons is coupled with a reduction in the density of striatal DA terminals and diminished levels in extracellular DA (Brooks et al., 1999). Moreover, treatment with PQ has also been shown to reproduce some of the motor deficits characteristic of PD phenotype (Brooks et al., 1999; Litteljohn et al., 2009). Furthermore, when considering the effects of other herbicides, PQ appears to be particularly more toxic. In a study assessing the toxicology of PQ and maneb pesticides, it was found that PQ is significantly more toxic than maneb because of its ability to

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inhibit proteasomal and autophagic pathways leading to an accumulation in alpha-synuclein inclusions (Wills et al., 2012). Lastly, PQ exposure is also accompanied by heightened inflammatory activity throughout the nigrostriatal system (Purissai et al., 2007).

Neuroinflammation and PD Pathology

Neuroinflammation is defined as the central nervous system's rapid immune response to perceived injury (Niranjan, 2014). Unlike the peripheral immune system, the CNS is largely devoid of leukocytes, due to the tight junctions of the BBB (Aloisi, 2001). As such, the CNS relies heavily on resident immune glial cells to mediate immune activity. Accordingly, neuroinflammation is more specifically defined as a state of reactive gliosis that results primarily from the chronic activation of glial cells, notably astrocytes and microglia, in response to CNS damage, disease, or repair (Niranjan, 2014). By this very nature, neuroinflammation is a consistent feature in neuropathological conditions, such as PD (Niranjan, 2014). While both astrocytic and microglial activity are involved in the neuroinflammation associated with PD, reactive microgliosis has proven to play a particularly profound role in PD pathogenesis (Block et al., 2007). Indeed, microglial cells have been shown to react readily to pro-oxidant conditions, such as environmental induced PQ neurotoxicity, by contributing further to the oxidative damage with the production of ROS (Vogt et al., 1998). Astrocytes, on the other hand, have demonstrated to be more resistant to toxin-induced oxidative stress (i.e. PQ toxicity) by responding with the induction of a variety of antioxidant systems (Olesen et al., 2008; Schmuck et al., 2002).

Microglia are the resident immune cells of the CNS and function primarily in immune surveillance and host defense. In the mature adult brain, microglia typically exist in a resting state characterized by a ramified morphology (Nimmerjahn et al., 2005). In this resting state, microglia monitor the microenvironment for extracellular signals indicative of CNS insult, which are readily

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detected by pattern recognition receptors (PRR) located on the glial cell surface (Sofroniew, 2009). These signals include molecules released by damaged or dead cells, pathogens or toxins (e.g. MPTP and PQ) entering the CNS via a leaky BBB, or molecules released by invading peripheral leukocytes or from the active glial cell themselves (Sofroniew, 2009). The detection of these signals results in the rapid proliferation and formation of enlarged microglial cell bodies, characterizing a morphologically active amoeboid glial form that is distinct from its quiescent ramified structure (Kreutzberg, 1996). These morphological changes are also accompanied by functional transformations, including increased metabolic activity and enhanced surface PRR (e.g. major histocompatibility complex and toll-like receptors (TLR)) expression (Block et al., 2007; Rock et al., 2004).

In their active state, microglia mediate the innate immune response. This is characterized by the clearance of toxic cellular debris, the production of inflammatory mediators (i.e. cytokines), the recruitment of peripheral leukocytes, the activation of the complement cascade, and antigen presentation (Jack et al., 2005; Town et al., 2005). In the presence of a CNS insult, microglia activation is critical for neuronal survival by directly eliminating harmful debris and by producing trophic and anti-inflammatory cytokines that aid in repair. However, due to the high responsiveness of active microglia, these cells can be readily over stimulated or dysregulated resulting in a sustained inflammatory state. This chronic inflammatory state is characterized by an excess production of pro-inflammatory cytokines (e.g. tumor necrosis factor-alpha (TNF- α) and interleukin-1 beta (IL-1 β)), further infiltration of leukocytes from the periphery), and the production and release of ROS (Liu & Hong, 2003). This rise in inflammatory factors and ROS levels can be detrimental to neuronal survival (Liu & Hong, 2003). As such, while beneficial in dealing with an initial threat, chronic activation of microglia can lead to progressive neurotoxic consequences that are hypothesized to promote the neuronal loss seen in neurodegenerative disorders such as PD (Block et al., 2007).

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The relation between neuroinflammation and dopaminergic neurodegeneration has been well documented over recent years. For example, post-mortem brain analyses of individuals exposed to the DA toxin, MPTP, when injected 16 years before death, revealed heightened microglial activation coupled with SNc DAergic degeneration (Langston et al., 1999). Similarly, mice treated systemically with the DA toxin, PQ, displayed concomitant nigrostriatal DAergic degeneration and microglial activation in the SNc (Purissai et al., 2007). In post-mortem brain analyses of PD patients, heightened microglial activation is observed throughout the midbrain, notably in the SN, the putamen, the hippocampus, the transentorhinal cortex, the cingulate gyrus, and the temporal cortex (Imamura et al., 2003). The SN, however, stands out as having a notably higher concentration of microglia compared to other regions, making it a particularly susceptible zone for microglial-induced neurotoxic damage (Kim et al., 2000). Moreover, as previously discussed, DA containing neurons are especially vulnerable to ROS damage due to the highly active redox state of DA (Zigmond et al., 2002). Thus, the colocalization of microglia with DA containing neurons in the SN particularly exposes vulnerable DAergic neurons to the potential oxidative stress that emerges with chronic microglial activation.

Given that microglia activation can be initiated from a variety of sources, it remains unclear whether microgliosis acts as a primary or secondary event in the DAergic degeneration of PD. For example, microglia activation may be exhibiting a primary role where exogenous toxins (e.g. PQ) directly activate microglia, generating the oxidative stress that initiates DA cell loss. Conversely, microglial may be serving a secondary role in response to DAergic dysfunction and loss. Indeed, damaged or dying DA neurons release signaling factors, notably, matrix metalloproteinase 3 (MMP3) (Kim et al., 2005), alpha-synuclein, (Zhang et al., 2005) and neuromelanin (Zecca et al., 2003), that induce ROS production from microglia, perpetuating reactive microgliosis and further facilitating DAergic degeneration. To better understand the relation between microglial-induced inflammation and

neurodegeneration, lipopolysaccharide (LPS) has been effectively applied as in inflammatory endotoxin in *in vitro* and *in vivo* models.

Lipopolysaccharide: An Inflammatory Stressor Implicated in the Study of PD. To date, lipopolysaccharide (LPS) is the best-studied trigger of a host inflammatory response and has become the prototype toxin for inducing inflammation (Alexander & Rietschel, 2001). LPS is a bacterial endotoxin found in the outer membrane of gram-negative bacteria. Although LPS is a microbial product, it is considered an endogenous compound to all mammalian species because of its presence in the gut flora (Marshall, 2005). Here, LPS containing gram-negative bacteria function as endosymbionts, where they exert multiple beneficial influences on immune homeostasis (Marshall, 2005). Under most conditions, the host is protected from the potential toxicity of LPS because of the thick mucus lining of the gastrointestinal track. However, under acute physiological stresses, such as vigorous exercise (Ashton, 2003), resumption of feeding after prolonged periods of deprivation (Ravindranath, 1997), exposure to multiple trauma (Buttenschoen et al., 2000), or gastrointestinal procedures, such as a colonoscopy (Berger et al., 1995), LPS can be readily absorbed by the epithelial lining of the gut. The consequential presence of endotoxins in the blood, a state referred to as endotoxemia, results in a pronounced immune response that can lead to septic shock at high enough doses. Similarly, in animals treated systemically with LPS, dose-dependent sickness behaviours (e.g. lethargy, curled body posture, ruffled fur, drooping eyes) and dose-dependent increases in plasma and central proinflammatory cytokine levels are observed (Gibb et al., 2011). Thus, whether absorbed endogenously by the host cells or administered exogenously, LPS exposure produces a robust inflammatory state.

When taken up by the host, LPS aggregates bind to plasma soluble protein, CD14, which enable it to interact specifically with the toll-like receptor 4 (TLR4) and accessory protein, myeloid differentiation protein 2 (MD2) (Lehnardt et al., 2003). TLR4 is apart of the TLR cell surface receptor

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family that responds primarily to LPS. It is found peripherally on leukocytes, dendritic cells, B cells, and macrophages, as well as centrally, on microglia. In microglia, TLR4 expression is upregulated in response to pathogens and neuroinflammation (McKimmie & Fazakerley, 2005). Specifically, when the LPS protein complex binds to TLR4, it initiates an intracellular kinase cascade that leads to the activation of the nuclear factor-kappa B (NF- κ B) transcription factor (Alexander & Rietschel, 2000). This resultant activates several hundred genes that contribute to the phenotype of an inflammatory response, including enhanced production and release of pro-inflammatory cytokines and ROS (Alexander & Rietschel, 2001). As such, TLR4 activation functions as the primary contributor to the microglial response to LPS, and has been shown to mediate LPS-induced neurotoxicity (Lehnardt et al., 2003),

Over the years, it has been shown that the inflammatory state of the peripheral environment contributes significantly to the inflammatory state of the CNS, and consequently, neurodegeneration. Notably, bacterial infections that lead to systemic inflammation are often associated with the exacerbation of clinical symptoms in individuals with neurodegenerative diseases (Perry et al., 2007; Wyss-Coray & Rogers, 2012). Moreover, this inflammation is accompanied by enhanced microglial activation (Perry et al., 2007; Hirsch et al., 2012). This relation between the peripheral and central environment has been replicated in animals using LPS. In deed, chronic systemic administration of LPS is capable of inducing microglial activation in mice (Kitazawa et al., 2005). Relevant to PD, this repeated exposure to LPS leads to particularly enhanced microglial activation in the SNc of mice (Bodea et al., 2014). Moreover, it has been shown that a single systemic injection of LPS is sufficient enough to cause a robust increase in the number of reactive microglia in the SNc of mice two days post treatment (Purisai et al., 2007). Complementing this, chronic or acute exposure to LPS is also associated with a rise in pro-inflammatory cytokine levels, notably TNF- α and IL-1 β , in the plasma and

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brain of mice (Bodea et al., 2014), mimicking the heightened pro-inflammatory levels seen in the brains of PD patients.

This enhanced neuroinflammatory state observed with a single systemic injection of LPS, is also accompanied by a significant loss in SNc-DAergic neurons in mice seven months post-treatment (Liu et al., 2008). Interestingly, chronic monthly LPS injections for five consecutive months produced not only a delayed, but a progressive loss in DA neurons; notably, a 40% neuronal loss at 9 months and 50% neuronal loss at 20 months of age (Liu et al., 2008). This degeneration was also accompanied by progressive behavioural deficits (Liu et al., 2008). Taken together, the LPS model recapitulates key elements of the neuropathological and clinical course of PD, highlighting the relevance of neuroinflammation in PD pathology.

Genetic Contribution to PD Pathology

The idea that genetics may play a role in PD pathology emerged from accumulating data on family medical history profiles. It was discovered that over 10% of PD cases are familial, where individuals have one or more first-degree relatives affected by the disorder, suggesting a role for genetic inheritance (Elbaz et al., 1999). Over the years these families have been instrumental in dissecting the genes involved in the pathogenesis of this disorder. To date, 18 loci of the human genome have been associated with PD (Bekris et al., 2010). Due to this advancement in genetic profiling, even PD cases that are regarded as idiopathic are beginning to reveal a genetic contribution. Notably, in a study that assessed over 500 families, up to 60% of idiopathic PD patients revealed genetic factors associated with PD etiology (Hamza & Payami, 2010). Among the known genetic mutations associated with PD, mutations in the leucine-rich repeat kinase 2 (LRRK2) gene are the most prevalent genetic cause of PD (Paisan-Ruiz et al., 2007).

LRRK2: A Gene Implicated in PD. Since the identification of the gene in 2004, LRRK2 mutations have been found to be associated with 10% of familial PD cases and 2% of idiopathic PD cases (Kruger, 2008). The LRRK2 gene, located at the *Park8* region on chromosome 12p11.2-q13, encodes for a 285-kDA multi-domain transmembrane protein with multiple functional and enzymatic domains. The functional domains include the ankyrin repeats, the leucine-rich repeats, and the WD40 repeats (Bae & Lee, 2015). Meanwhile the enzymatic domains consist of the Ras of complex (ROC) GTPase domain, the carboxyl-terminal of ROC (COR) domain, and the kinase domain (Bae & Lee, 2015). Although LRRK2 expression is highest in the kidneys, lungs and lymph nodes (Biskup et al., 2007), LRRK2 is also found in several brain regions implicated in PD neuropathology (e.g. SN, caudate putamen, striatum, and cerebral cortex) (West et al., 2014; Westerlund et al., 2008). In the brain, LRRK2 has been reported to regulate microglial activity (Moehle et al., 2012), mediate neuronal outgrowth and guidance (Macleod et al., 2006), coordinate vesicular storage and mobilization (Piccoli et al., 2011), and modulate mitogen-activated protein kinase (MAPK) dependent autophagic (Bravo-San Pedro et al., 2013) and apoptotic processes (Chen et al., 2012). *In vivo* studies have shown that these physiological functions of LRRK2 are linked to the enzymatic functions of the GTPase and kinase domains (West et al., 2007). Indeed, pathogenic mutations that are associated with PD are concentrated within these domains (Biskup & West, 2009).

The most common pathogenic mutation associated with PD is the G2019S amino acid substitution occurring in the kinase domain of LRRK2 (Sundel et al., 2012). The G2019S mutation accounts for approximately 5% of familial PD cases and 1-2% of idiopathic PD cases worldwide (Berg et al., 2005). Interestingly, these odds vary drastically depending on the population. For example, the G2019S mutation is prevalent in 1-2% of PD patients in the United Kingdom and United States, 10% of PD patients of Ashkenazi Jewish decent, and 39% of PD patients of the North African Berber population (Healey et al., 2008a). Among individuals carrying the G2019S mutation, age appears to be

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the greatest risk factor for developing PD, with a 28% penetrance at 59 years of age, 51% penetrance at 69 years of age, and 74% penetrance at 79 years of age (Healey et al., 2008b). Based on these etiological findings, recent research has been aimed at addressing the molecular actions of the G2019S mutation.

The molecular effect of the G2019S mutation is to enhance the enzymatic kinase capacity of LRRK2 (Li et al., 2010). Indeed, it has been shown that the G2019S mutation facilitates access of substrates to the kinase domain, resulting in a 2.5-fold increase in autophosphorylation and 3-fold increase in phosphorylation of substrates (West et al., 2005). Interestingly, the kinase domain of LRRK2 has high sequence homology to the mixed lineage kinases (MLKs), which are members of the mitogen-activated protein kinase (MAPK) family (Mata et al., 2006). MAPK kinases consists of three well-known subfamilies; the MAPK/extracellular signal-regulated kinase (ERK) pathway that functions primarily in cell proliferation and survival, the p38 MAPK pathway, which responds readily to inflammatory cytokines and environmental stressors, and the MAPK/c-Jun-N-terminal kinase (JNK) pathway, which predominately mediates cellular apoptosis (Mata et al., 2006). The activation of the MAPK/JNK pathway has been linked to PD pathology (Wang et al., 2004). For example, in a postmortem analysis of PD brains, active phospho-JNK staining was detected in neurons throughout the midbrain region and, more specifically, in neurons that were in close proximity to alpha-synuclein containing LB and LN inclusions (Ferrer et al., 2001). Interestingly, recent reports have shown that the enhanced kinase capacity of G2019S-LRRK2 is associated with heightened activity of the MAPK/JNK pathway, supporting the close resemblance between MLKs and the LRRK2 kinase domain. Indeed, it was found that 12 month-old mice containing the G2019S mutation displayed increased levels in active phospho-MKK4 protein and active phospho-JNK protein in the SN (Chen et al., 2012). This subsequently induced a downstream increase in the protein levels of caspase-9, -8, and -3 in the SN,

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which have known functions in promoting neuronal death by activating on intrinsic and extrinsic apoptotic pathways (Chen et al., 2012).

To further add to the pathology induced by this mutation, G2019S-LRRK2 is also linked to heightened oxidative stress. Notably, in a recent study that assessed the oxidative state of the SN in transgenic rats containing the G2019S mutation, it was found that G2019S rats had significantly higher levels in nitrotyrosine and iNOS within DA neurons of the SN compared to wild-type (WT) controls (Lee et al., 2015). This increase in oxidative species may be attributable to disruptions in mitochondrial activity. In fact, it has been shown that the G2019S-induced increase in kinase capacity results in mitochondrial uncoupling via upregulation of uncoupling protein (UCP) expression (Papkovskaia et al., 2012). This increase in UCP expression decreases the membrane potential, hindering ATP production and facilitating ROS generation. These data are consistent with previous reports indicating decreased mitochondrial membrane potential and total ATP levels in fibroblasts extracted postmortem from PD patients with the G2019S mutation (Mortiboys et al., 2010).

Although little is known on the specific role the G2019S mutation plays in the inflammatory response, LRRK2 itself is tightly linked to immune processes. Indeed LRRK2 is expressed in peripheral immune cells, notably B cells, monocytes, and dendritic cells, and this expression is upregulated upon interferon-gamma (IFN- γ) stimulation (Gardet et al., 2010). This upregulated LRRK2 expression results in increased activity of NF- κ B pathways promoting a proinflammatory phenotype (Gardet et al., 2010). Moreover, LRRK2 is recruited near pathogens in response to bacterial induced infection with the bacteria, *S.typhimurium* (Gardet et al., 2010). Interestingly, knocking down LRRK2 expression results in higher levels of surviving, *S. typhimurium*, indicating that LRRK2 contributes to the antibacterial activity of macrophage cells (Gardet et al., 2010). Furthermore, LRRK2 has also been shown to regulate immune responses in microglial cells of the brain (Moehle et al.,

2012). Indeed, central infusions of LPS into the SNc results in significantly enhanced LRRK2 expression in active microglia at the site of injection in WT mice (Moehle et al., 2012).

The influence of the G2019S mutation on DAergic degeneration in the SNc is inconsistent to say the least. In a longitudinal study that analyzed TH-positive cell counts in the SNc of mice with or without the G2019S mutation at 6 months, 12 months, and 18-20 months, there was no difference found in the number of TH-positive cells between the groups (Li et al., 2010). Conversely, in another study that assessed 12-16 month-old mice, G2019S mice displayed an approximate 30% reduction in TH-positive DA cells in the SNc compared to WT controls (Chen et al., 2012). Similar discrepancies are also apparent in regard to G2019S related changes in motor behaviour. For example, aged mice overexpressing G2019S have been associated with both a hyperkinetic phenotype (Lango et al., 2014) and a hypokinetic phenotype (Chen et al., 2012; Chou et al., 2014). Meanwhile it has also been shown that the overexpression of G2019S produces no deficits in motor behaviour in aged mice (Li et al., 2010) and in aged rats (Lee et al., 2015). More consistent reports are seen in respect to extracellular striatal DA content, where the G2019S mutation is associated with an age dependent reduction in evoked DA release (Chou et al., 2014; Li et al., 2010). Nonetheless, this discrepancy in the data suggests that age alone is not sufficient enough to significantly increase the penetrance of G2019S. Additional environmental factors are likely necessary for precipitating the pathology and for exposing the role of G2019S in PD.

Sex Differences in PD Pathology

In recent years, sex differences have received increasing recognition as a prominent factor affecting the incidence and nature of PD etiopathology. At all ages and among all nationalities the male sex represents a significant risk factor, with a male to female rate of incidence varying from 1.37–3.7 (Gillies, 2014). Clinically, the age of onset in women is approximately two years later (Alves et al.,

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2009) with women expressing an overall milder PD phenotype, particularly during the early stages of the disease (Miller & Cronin-Golomb, 2011). Molecularly, post mortem brain analyses have revealed that genes involved in PD pathogenesis (e.g. PINK1) are upregulated in nigral DA neurons in men, while genes involved in signal transduction and neuronal maturation are upregulated in nigral DA neurons in women (Cantuti-Castelvetri et al., 2007). Moreover, in animal toxin models of PD, greater loss in striatal DA levels are observed in male rodents treated with 6-hydroxydopamine (6-OHDA) (Gillies et al., 2004; McArthur et al., 2007; Moroz et al., 2003) and MPTP (Liu & Dluzen, 2007) when compared to females. Similarly, it has been shown that female mice treated chronically with PQ were protected from PQ-induced deficits in striatal DA levels and locomotor activity (Litteljohn et al., 2011). These data opposed previous findings where male mice exposed to the same PQ treatment regime displayed significant loss in striatal DA content and deficits in locomotion when compared to saline controls (Li et al., 2005; Litteljohn et al., 2009). When trying to understand the cause for this sex discrepancy, a number of factors come into play, including differences in sex hormones, sex chromosomes, lifestyle, and so forth. Sex hormones, however, are by far the most important factors for influencing structural and functional sexual differentiation in the brain (Gillies et al., 2014). As such, sex hormones are likely the driving forces for sex differences in disease vulnerability.

Differences in PD susceptibility and in PD pathology as it relates to estrogen levels have been well documented over the years. For example, at menses, when cyclic estrogen levels are at their lowest, PD symptom severity has been shown to worsen in women (Quinn & Morsden, 1986). Additionally, women who undergo a bilateral ovariectomy prior to menopause (i.e. eliminating most peripheral sources of estrogen) have an increased risk for developing PD (Rocca et al., 2008). Moreover, estrogen replacement therapy has been shown to relieve PD symptoms when given at early stages in the disease (Benedetti et al., 2001) and can even decrease the risk of developing PD (Liu & Dluzen, 2007). Correspondingly, in animal toxin models of PD, striatal DA lesions caused by 6-OHDA

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infusions are far greater in ovariectomized rats compared to gonad intact females (Ferrez et al., 2008). This effect can be reversed when estrogen is restored to physiological levels (Ferrez et al., 2008). In a study that treated mice with MPTP during different phases of the estrus cycle, it was found that MPTP administered during proestrus, when estradiol levels are maximal, produced a smaller loss in striatal DA content compared to MPTP administered during diestrus, when estradiol levels are lowest (Dluzen & Horstink, 2003). *In vitro* reports that looked at the role of estradiol in neuroinflammation showed that incubating microglial cultures in estradiol prevents the DA cell loss that is otherwise induced with LPS (Liu et al., 2005). Additionally, ovariectomized rats display a 50% increase in DAT levels, as well as extracellular DA depletion, with both effects reversed with estradiol treatment (McArthur et al., 2007). In this regard, estradiol may be exerting its protective effects by suppressing DAT levels. Indeed suppressing DAT expression will repress DA reuptake and consequently intraneuronal DA metabolism, which leads to ROS production. Taken together, these data suggests that estradiol exhibits a global neuroprotective effect in females.

Conversely, estrogen does not appear to exert the same degree of pro-dopaminergic and neuroprotective effects in males. While some studies have reported a modest attenuation in toxin-induced striatal DA loss in male rodents treated with estradiol (Ramirez et al., 2003; Shugrue, 2004), others have failed to replicate this beneficial effect of estradiol on DA signaling in males (Becker et al., 1999; McArthur et al., 2007; McDermott et al., 1994). The inability of estrogen to exert the same degree of neuroprotective effects in males may be explained by brain defeminization, which is hypothesized to lead to a loss in capacity to respond to estrogens (McCarthy, 2008). Perinatally, males experience a surge in testosterone levels, exposing the malleable perinatal brain to high levels of estrogen when testosterone is aromatized, which it readily is. This early exposure to estrogen in males is thought to result in a loss of capacity to respond to estrogen later in life, whereas females retain this

ability. As such, the beneficial effects of estrogen appear to be limited to females, thus providing one possible mechanistic account for the sex differences observed in PD.

A Multi-Hit Hypothesis of PD Pathology

As previously stated, it is generally accepted that PD is a multifactorial disease arising from a constellation of environmental and inflammatory factors, in conjunction with a genetic vulnerability (Betarbet et al., 2006; Kelada et al., 2006). This multi-hit view is supported by the simple fact that animal models exposed to a single hit alone (e.g. PQ alone, LPS alone, genetic mutation alone) have not been successful at recapitulating the entire neuropathological and clinical phenotype of PD. As such, combinatorial study of these different models are more recently being addressed and are proving to provide better insights into the etiopathogenesis of PD.

Notably, it has been shown that systemic LPS treatment followed by PQ markedly enhances nigrostriatal DA cell loss when compared to LPS or PQ alone (Purisai et al., 2007). Similarly, intracranial LPS infusions into the SNc have been shown to sensitize DA neurons to the neurodegenerative effects of chronic systemic PQ treatment commencing 2 days later (Mangano & Hayley, 2008). Taken together, this data suggests that inflammatory priming renders DA neurons more susceptible to environmental toxins by heightening glial and immune activity. In fact, a recent finding on the mechanism through which PQ is taken up by DAergic neurons supports just that. As previously stated, in its native divalent cation form, PQ is not a substrate for DAT (Rappold et al., 2011). Indeed, Rappold and colleagues (2011) found that when PQ is oxidized to its monovalent form by a reducing agent such as molecular oxygen or NADPH oxidase, PQ becomes a substrate for DAT where it can readily be taken up by DAergic neurons. Thus, according to these findings, PQ must be oxidized extracellularly before exerting cytotoxic effects on DAergic neurons. Interestingly, microglia rapidly up-regulates surface NADPH oxidase when activated (Rock et al., 2004), providing a means for which

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PQ can be extracellularly oxidized and supporting the notion that neuroinflammation sensitizes environmental induced neurotoxicity.

In PD, the penetrance of genetic mutations is quite low suggesting the necessity of environmental insults. Accordingly, it has been shown that chronic treatment with PQ in mice containing mutations in the PD relevant DJ-1 gene facilitates the expression of motor deficits (Yang et al., 2007). Such deficits in behaviour are only sparingly observed in WT mice treated with PQ or in aged DJ-1 mutated mice (Yang et al. 2007). Moreover, in a recent report, it was found that when G2019S transgenic mice were treated with lactacystin, a proteasome inhibitor, they displayed significantly greater nigrostriatal DAergic degeneration and greater decline in striatal DA content compared to G2019S and WT controls (Xiao et al., 2015). Moreover, this effect was more pronounced at 12 months compared to 5 months, indicating that the environmental toxin was sufficient at enhancing the penetrance of G2019S in an age-dependent manner (Xiao et al., 2015).

Collectively, these studies advocate for the necessity of multiple hits to bring forth a more robust pathological effect. In doing so, these multi-hit models provide greater insights into the synergistic effects of environmental and genetic insults allowing for better understandings of the mechanisms underlying PD pathology. The aim of the current research thesis is to explore these environmental and genetic synergistic actions in G2019S-LRRK2 mutated mice using an LPS+PQ treatment regimen. Furthermore, due to the robust effect of sex differences documented in PD pathology, basic sex effects will also be examined. We hypothesize that LPS inflammatory priming will potentiate the neurotoxic effects of chronic PQ treatment, resulting in heightened inflammation and neurodegeneration of the DAergic nigrostriatal system. Moreover, we posit that these neuropathological features will be accompanied by the expression of motor deficits, indicative of a parkinsonism-like phenotype. Furthermore, we suspect that genotype and sex effects will emerge in the

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form of more pronounced inflammation, degeneration, and deficits in motor behaviour among the G2019S mutated mice and male mice.

Method

Animals

Male mice hemizygous for the bacterial artificial chromosome (BAC) G2019S-LRRK2 transgene were purchased from Jackson Laboratory. Mice harbouring this mutant gene are viable, fertile, and display no significant physical or behavioural abnormalities. Mice containing this mutation overexpress the mouse G2019S-LRRK2 mutant protein in the cerebral cortex, striatum, SN, internal capsule, and hippocampus at an approximately 6-8 fold greater level than endogenous mouse LRRK2 (West et al., 2014). These purchased male mice were then bred in house with female C57BL/6J wild-type (WT) mice producing a generation of hemizygous G2019S-LRRK2 mice and WT mice. Eighty male and female adult mice (8-10 weeks old) hemizygous for the G2019S-LRRK2 mutation ($n=40$) and WT-LRRK2 littermate controls ($n=40$) were used.

Animals were single housed in transparent polypropylene shoebox cages (27cm x 21cm x 14cm) with corncob bedding. All animals were acclimated to the vivarium for a one-week period prior to the initiation of procedures. Mice were kept on a 12 hour light/dark cycle (lights on at 8:00am) in a colony room maintained at a constant temperature of 21°C with a humidity of 50%. Animals were given water and Ralston Purina mouse chow *ad libitum*. All procedures conformed to the guidelines of the Canadian Council for the Use and Care of Animals in Research and were approved by the Carleton University Committee for Animal care.

Treatment Administration and General Experimental Procedures

Male and female (random, undetermined estrous cycle stage) G2019S and WT mice were randomly assigned to either a lipopolysaccharide (LPS)-paraquat (PQ) treatment regimen or a control saline condition (*refer to figure 1 for group assignment*). Animals in the LPS+PQ condition received a single 5 mg/kg intraperitoneal (i.p) injection of LPS (from *Escherichia coli* 055:B5; Sigma-Aldrich,

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Oakville, ON, Canada) followed by six treatments with PQ (1,1'-dimethyl-4,4'-bipyridinium dichloride; Sigma-Aldrich, Oakville, ON, Canada) injected i.p. at a dose of 10 mg/kg. A single systemic injection of LPS at the aforementioned dose has been shown to produce delayed DA cell loss in the SN of male C57BL/6J mice (Liu et al., 2008). Similarly, chronic exposure to PQ at the aforementioned dose has also been shown to produce DA cell loss in the SNc and striatum of C57BL/6J mice, as well as deficits in motor activity (*reviewed in* Litteljohn et al., 2010). The first PQ treatment was administered 48 hours following the LPS treatment, when microglia are in their most active state (Mangano & Hayley, 2009). The subsequent five PQ treatments were administered every three days over a three-week period. Animals in the control condition received i.p. injections of pyrogenic free physiological saline (Sigma-Aldrich, Oakville, ON, Canada) of equivalent volume in place of the LPS and PQ treatments. All treatments were administered between 9:00 and 11:00am.

Baseline motor behaviours were obtained 24 hours prior to LPS/saline treatment in order to address any potential pre-existing sex and genotype differences. Motor behavioural deficits were then tested for using Micromax and Catwalk apparatuses one day following the first, third, and fifth PQ/saline treatment, in order to characterize the temporal profile of LPS+PQ induced behavioural pathology. Motor deficits were also examined using Rotarod two days following the sixth and final PQ injection. All motor behaviours were assessed either three days following the LPS treatment and/or one day following the subsequent PQ treatments in order to avoid the potential acute toxic effects of LPS and PQ. All motor behaviour assessments occurred between 8am and 4pm. Sickness behaviours were recorded throughout the study to monitor animal needs and to address any acute effects of LPS and PQ treatment. Animals were euthanized three days following their last PQ/saline treatment and perfused for tissue extraction (*refer to Figure 2 for an experimental timeline*).

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Figure 1. Group assignment. Group assignment of mice based on sex (male or female) and genotype (WT or G2019S) and random assignment into one of two treatment conditions (control saline treatment or the combined LPS+PQ treatment). WT: mice wild-type for LRRK2; G2019S; mice bearing the G2019S mutation in LRRK2; LPS+PQ; lipopolysaccharide + paraquat treatment regimen.

Figure 2. Experimental Timeline. Course of treatment and schedule of motor behavioural testing. LPS: lipopolysaccharide; PQ: paraquat.

Behavioural Assessments

Sickness behaviour and weight. Sickness was closely monitored at 1-hour intervals over a 6-hour period following the LPS injection and again 24 hours post LPS treatment. Sickness scores were obtained using a 4 point rating scale, where a score of 1 indicates no sickness, a score of 2 refers to lethargy (i.e. diminished locomotion and exploratory activities), a score of 3 refers to significant

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lethargy with a curled body posture and the presence of either ptosis (i.e. drooping eyelids) or piloerection (i.e. ruffled and greasy fur, typically at the neck), and lastly a score of 4 refers to completely diminished movement and poor responsiveness, curled body posture, ptosis, and piloerection. This procedure was previously found to provide less than 10% variability between raters blind to the treatment mice received and was highly correlated with other methods of scoring sickness (e.g. assessing severity of each symptom independently) (Gandhi et al., 2007). All scores were obtained by the same blind rater. Animals that reached endpoint were euthanized and given a score of 5. Animals receiving a sickness score of 2 or greater were monitored and treated accordingly (i.e. placed on a heat pad, administered hydrogel and mush food, and treated with subcutaneous injections of saline). Sickness scores were also obtained prior to behavioural tests to ensure no sickness interference effects that may remain post LPS administration or emerge in response to PQ treatments. Animals that displayed sickness behaviours prior to testing were removed from the motor behavioural assessment. Additionally, weight was recorded at baseline, prior to each treatment, and 24 hours following each treatment.

Spontaneous home-cage locomotor activity. Spontaneous locomotor activity was measured over a complete 12-hour dark cycle using a Micromax infrared beam-break apparatus (Accuscan Instruments, Columbus, OH, USA) located exterior to the animal's home cage. The number of breaks in the infrared beam resulting from the animal's movement in their cage was recorded as a measure of spontaneous locomotor activity in a familiar environment. This spontaneous locomotor activity was measured at four time points; at baseline, one day prior to LPS/saline treatment and then one day following the first, third, and fifth PQ/saline treatments. A reduction in spontaneous movement may be indicative of deficits in motor activity. As such, it was hypothesized that animals in the LPS+PQ condition will display, on average, reduced spontaneous activity compared to saline controls, with a possible worsening in activity occurring overtime. Moreover, we suspected that potential genotype and

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sex effects will emerge in the form of more pronounced reductions in activity among the G2019S mutant mice and male mice.

Gait performance. Gait (i.e. pattern of movement of the limbs) was assessed using Catwalk XT Gait Analysis System (Nodulus Information Technology, Asheville, NC, USA). Mice were allowed to freely ambulate along an illuminated glass plate within a confined corridor and footprints were recorded with a high-speed camera. Gait analyses were assessed at four different time points; at baseline, one day prior to LPS/saline treatment and then one day following the first, third, and fifth PQ/saline treatments. At each testing, animals were required to complete three compliant runs as determined by the software. For each variable, the data of each run was averaged into a single score for each animal.

Variables that are of particular interest include Maximum Contact Area (i.e. maximum area of a paw that comes into contact with the glass plate) Stride Length (i.e. distance between successive placements of the same paw), Regulatory Index (i.e. ratio of the number of normal step sequence patterns relative to the total number of paw placement), and Base of Support (i.e. average width between either the front paws or the hind paws). Animals that are displaying parkinsonism-like motor deficits will likely show increases in Maximum Contact Area where greater surface area of the limb is required for movement. Reductions in stride length would indicate shuffling-like steps, which may be characteristic of a parkinsonism-like trait. Decreases in the Regulatory Index would indicate a disruption in overall walking pattern, suggesting a parkinsonism-like motor deficit. Lastly, increases in Base of Support would indicate an increase in width distance between the paws, implying instability in gait. We hypothesized that animals in the LPS+PQ condition will display, on average, greater deficits in gait, with possible worsening in gait occurring overtime, when compared to saline controls. Moreover, we suspected that potential genotype and sex effects will emerge in the form of more pronounced deficits in gait among the G2019S mutant mice and male mice.

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Rotarod. Rotarod training and testing was performed to assess deficits in motor coordination and balance. To do this, an EzRod system connected to test chambers (44.5cm x 14cm x 51cm; Accuscan Instruments) consisting of a motorized rod (1cm in diameter) was used. Rotarod training and testing occurred over three consecutive days following the final PQ treatment. On day one and two animals were trained to stay on the motorized rod moving at a constant speed of 12 rpm and 22 rpm, respectively, for a 5-minute period. Animals that fell off were quickly placed back on the rod. This was repeated twice for a total of three 5-minute training sessions spaced over 1-hour intervals, so as to avoid the interference of animal fatigue. On day three, animals were tested for their ability to stay on an accelerating rod that gradually increased from 4 to 44 rpm over a 5-minute period. Animals were assessed on their ability to stay on the rod, noting down the time at which they fell (if they fell). Time spent on rod is hypothesized to be an indication of motor coordination and balance ability, where less time spent on the rod would suggest deficits in these behaviours. Similar to training, this procedure was repeated twice, for a total of three testing sessions spaced over 1-hour intervals. The lowest time spent on the rod was removed and the remaining times were averaged giving a single score for each animal. This was done to account for the possibility of the animal accidentally falling off the rod. We hypothesized that animals in the LPS+PQ condition will spend less time, on average, on the rod compared to saline controls. Moreover, we suspected that potential genotype and sex effects will emerge in the form of significantly less time spent on the rod among the G2019S mutant mice and males mice

Perfusions and Tissue Extraction

Animals were anesthetized with sodium pentobarbital and subsequently euthanized via transcardial perfusion with 0.9% saline followed by fixation using 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS), pH 7.2. The brains were dissected and post-fixed in 4% PFA overnight.

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Tissues were then stored in 0.1 M phosphate buffer (PB), pH 7.4, with 10% sucrose at 4°C for 24h, followed by storage in 0.1 M PB with 30% sucrose at 4°C for an additional 48h. Tissues were then flash frozen and stored at -80°C.

Immunohistochemistry

Brain tissue was sliced using a Shandon AS620 Cryotome (Fisher Scientific, Ottawa, ON) and coronal sections (40um-thick for the striatum and 60um-thick for the SNc) were obtained and floated in 0.1M phosphate buffer, pH 7.4+ azide. Every second section was selected for tyrosine hydroxylase (TH) immunohistochemistry and CD68 immunohistochemistry, respectively. TH is the rate-limiting enzyme responsible for catalyzing the synthesis of DA, and is therefore used as a marker for DA containing neurons. CD68 is a glycoprotein found in the cytoplasm of macrophages, and can therefore be used a marker of microglial cells and morphology.

For TH and CD68 immunohistochemistry, striatal and SNc sections were first incubated in 0.3% hydrogen peroxide for 30 minutes and blocked for 1 hour in 5% normal goat serum (NGS) and 0.3% triton-X, with 0.1 M PBS, pH 7.2 washes in between. Blocker was then removed and half of the striatal and SNc sections were incubated in anti-mouse TH at a dilution of 1:2000, while the other half of the striatal and SNc sections were incubated in anti-rat CD68 at a dilution of 1:2000. Sections were kept at room temperature overnight. The following day, sections were rinsed with PBS. SNc-TH sections were then incubated in anti-mouse HRP at a dilution of 1:200 at room temperature for 4-6 hours. Meanwhile, striatal-TH sections were incubated in anti-mouse IgG secondary at a dilution of 1:500 and striatal-CD68 and SNc-CD68 sections were incubated in anti-rat IgG secondary at a dilution of 1:500 at room temperature for 2 hours. Striatal-TH, striatal-CD68, and SNc-CD68 underwent an additional incubation in HRP at a dilution of 1:1000 for 2 hours. All sections were then rinsed with PBS and a DAB reaction was performed in order to colour the peroxidase and allow for the

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visualization of HRP. To do this, sections were incubated in a DAB solution for 5 minutes and reacted with 30% hydrogen peroxide for an additional 10 minutes. Sections were washed with PBS to stop the reaction and subsequently mounted on slides and allowed to dry overnight. The following day, sections were dehydrated with alcohol and cleared and coverslipped using DPX.

Immunohistochemical Assessment

TH quantification. Stereological analyses of SNc sections were used to determine DA degeneration (*previously described in* Mangano et al., 2011). Using MicroBrightField Inc. StereoInvestigator software, the SNc was outlined under a 2.5x magnification and TH-positive neurons were counted using a 60x oil immersion objective lens. The SNc was sampled in a systematic random fashion according to the optical fractionator method outlined in the software. Cells were quantified in 3-dimensional counting frames using a counting grid size of 90x90 μm and a counting frame size of 60x60 μm with a 15 μm dissector height and 3 μm upper and lower guard zones. Total SNc TH-positive cells were quantified. All analyses were conducted by the same individual blind to the experimental conditions. It was hypothesized that animals in LPS+PQ condition will have significantly less TH-positive cell counts, on average, compared to saline controls. Moreover, we suspected that potential genotype and sex effects will emerge in the form of more pronounced SNc DAergic degeneration occurring among the G2019S mutant mice and male mice.

CD68 quantification. Microglial reactivity was semi-quantitatively and blindly evaluated using a previously established rating scale that categorizes the degree of morphological changes in glial structure (Mangano & Hayley, 2008). The rating scale ranged from 0 to 3, where 0 refers to a resting glial (i.e. highly ramified thin processes), 1 refers to an intermediate reactive state in which less than 20 cells/SNc could be considered moderately activated (i.e. thickened, short processes with a compact soma), a rating of 2 was given when the majority of glial cells are intermediately activated with

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occasional highly activated cells (i.e. amoeboid macrophage-like appearance, being spherical shape and lacking processes), and a rating of 3 was given when a large number of cells display the most highly activated amoeboid shape. It was hypothesized that animals in the LPS+PQ condition will display, on average, heightened microglial activity with higher scores on the rating scale, compared to saline controls. Moreover, we suspected that potential genotype and sex effects will emerge in the form of even greater inflammatory activity occurring among the G2019S mutant mice and male mice.

Statistical Analyses

All data were analyzed using a 2 (sex; male vs. female) X 2 (genotype; WT vs. G2019S) X 2 (treatment; saline vs. LPS+PQ) between-subjects analysis of variance (ANOVA) design. When there was an interaction effect, three-way ANOVA's were followed by Fisher's post-hoc simple comparisons ($p < .05$). Data are presented in the form of mean $\pm$ standard error mean (mean $\pm$ -SEM). All data were evaluated using a StatView (version 6.0) statistical software package available from the SAS Institute, Inc., and differences were considered statistically significant when $p < .05$.

Results

Behavioural Analysis

Sickness behaviour and weight. A three-way analyses of variance (ANOVA) was conducted to assess the effect of sex, genotype, and LPS treatment on sickness behaviour and weight. Two hours post-LPS/saline treatment, a significant sex by treatment interaction was found, $F(1, 84) = 4.41, p < .0388$. Subsequent simple analyses revealed that, irrespective of genotype, male mice treated with LPS ($M = 1.37, SEM = .10$) displayed significantly greater sickness behaviours compared to female mice exposed to LPS ($M = 1.17, SEM = .07$), (*Figure 3A*). Sickness behaviours were still significantly elevated in LPS treated mice at six $F(1, 84) = 148.54, p < .001$ and 24 hours $F(1, 84) = 58.27, p < .001$ post-LPS treatment (*Figure 3*). All ratings of sickness were absent by 48 hours post-treatment.

Concomitant with the sickness symptoms was a significant loss in body weight 24 hours post-LPS exposure. The ANOVA revealed a main effect of treatment, $F(1, 82) = 414.747, p < .001$, where mice exposed to LPS ($M = -2.07, SEM = .60$) showed significantly greater weight loss compared to mice treated with saline ($M = -.01, SEM = .13$) (*Figure 4*). Weight loss occurred irrespective of sex or genotype and recovered by 72 hours post-treatment.

Sickness symptoms and weight were also recorded throughout the experiment in order to avoid any interference of such symptoms when assessing motor behaviours. In total, four mice reached end-point by the 5th or 6th PQ treatment, indicating acute toxicity and were hence, excluded from analyses of motor behaviours.

Figure 3. *Sickness Behaviour Varied as a Function of Treatment and Sex.* Overall, LPS exposure induced significant sickness symptoms in all mice at two (A), six (B), and 24 (C) hours post treatment. Moreover, these effects were slightly more pronounced in male mice compared to female mice at two hours (A). Data are expressed as mean $\pm$ SEM. * $p < .05$, ** $p < .01$ main effect of treatment. † $p < .05$ sex by treatment interaction. LPS: lipopolysaccharide; WT: mice that are wild-type for LRRK2; G2019S: mice that bear the G2019S mutation in LRRK2.

Figure 4. *Weight Loss Varied as a Function of Treatment.* Irrespective of sex and genotype, mice treated with LPS displayed marked and statistically significant weight loss 24 hours post-treatment

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compared to mice exposed to saline. Data are expressed as mean $\pm$ SEM. $*p < .01$. LPS:

lipopolysaccharide; WT: mice that are wild-type for LRRK2; G2019S: mice that bear the G2019S mutation in LRRK2.

Spontaneous home-cage locomotor activity. A three-way ANOVA was conducted to assess the effect of sex, genotype, and treatment on spontaneous home-cage motor activity. Animals that displayed visible signs of sickness at testing time were removed from these analyses so as to avoid sickness interference effects on motor activity. At baseline, the ANOVA revealed that sex and genotype did not significantly affect spontaneous home-cage locomotor activity (*data not shown*). However, LPS+PQ treatment significantly affected spontaneous home-cage locomotor activity at all three time-points assessed. Indeed, at the three time points (24 hours after PQ injections 1, 3, and 5) there were significant main effects of treatment, $F_s(1, 78) = 22.53, 5.04, \text{ and } 10.94$ $p_s < .05$, such that mice that received LPS+PQ displayed significantly lower spontaneous home-cage locomotor activity compared to mice that received saline (*Figure 5*). Sex and genotype, however, did not significantly influence spontaneous home-cage locomotor activity at any of the three time points.

Figure 5. *Spontaneous Home-Cage Locomotor Activity Varied as a Function of Treatment.* Irrespective of sex and genotype, mice exposed to the combined LPS+PQ treatment regimen displayed significantly

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reduced spontaneous home-cage locomotor activity compared to mice treated with saline at time points one (A), two (B), and three (C). Data are expressed as mean $\pm$ SEM. $*p < .05$. LPS+PQ:

lipopolysaccharide + paraquat; WT: mice that are wild-type for LRRK2; G2019S: mice that bear the G2019S mutation in LRRK2.

Gait performance. A three-way ANOVA was conducted to assess the effect of sex, genotype, and treatment on gait as measured by catwalk. Animals that displayed visible signs of sickness during testing were removed from catwalk analyses so as to avoid sickness interference effects on performance of the gait task. Once again, at baseline, the ANOVA revealed that sex and genotype did not significantly affect gait performance (*data not shown*). Likewise, no significant three-way interactions were present for any of the measures. However, following the 5th PQ/saline exposure, a significant Sex by Treatment interaction was evident for the average ambulatory width of distance between the front paws, $F(1, 62) = 15.47, p < .001$. Specifically, further comparisons revealed that female mice treated with LPS+PQ displayed significantly *greater* ambulatory width between the front paws when compared to saline controls ($p < .05$). Conversely, males treated with LPS+PQ displayed, on average, significantly *reduced* ambulatory width between the front paws when compared to saline controls ($p < .05$) (*Figure 6*). These findings suggest that the combined LPS+PQ treatment regimen results in opposing gait responses for males and females. The greater splay in forelimbs in females treated with LPS+PQ is likely indicative of a struggle in ambulation and is consistent with previous reports of deficits in gait (Mangano & Hayley, 2009). The narrowing of width between the forelimbs in males treated with LPS+PQ is presumably a more challenging mode of ambulation and may also be indicative of gait deficits but which may be qualitatively different.

Although the Sex by Treatment effects did not significantly interact with genotype, it was clear that the G2019S mice displayed the most prominent gait changes. Moreover, a significant main effect for genotype was evident, $F(1, 62) = 5.36, p = .024$, such that G2019S animals ($M = 7.32, SEM = 1.25$)

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displayed significantly greater ambulatory width between the front paws compared to WT animals ($M = 5.31$, $SEM = .74$) (Figure 6). Similarly, a main effect of genotype, $F(1, 66) = 6.746$, was also found for the spreading of the hind paws, such that G2019S animals ($M = 15.39$, $SEM = 2.88$) had significantly greater ambulatory width between the hind paws compared to WT animals ($M = 10.58$, $SEM = 1.35$), irrespective of sex and treatment (Figure 7). Thus the G2019S mutation basally may influence gait, but not necessarily sensitivity to toxicant challenge.

Figure 6. *Gait Performance Varied as a Function of a Sex by Treatment Interaction and as a Function of Genotype.* The average ambulatory width between the front paws varied as a function of a Sex by Treatment interaction, such that female mice exposed to LPS+PQ had significantly greater ambulatory width between the front paws compared to saline controls. Conversely, male mice exposed to LPS+PQ had significantly reduced ambulatory width between the front paws compared to saline controls. Additionally, G2019S animals overall displayed greater ambulatory width between the front paws compared to WT animals. Data are expressed as mean $\pm$ SEM. * $p < .01$ simple comparisons of treatment, † $p < .05$ main effect of genotype. LPS+PQ: lipopolysaccharide + paraquat; WT: mice that are wild-type for LRRK2; G2019S: mice that bear the G2019S mutation in LRRK2.

Figure 7. *Gait Performance Varied as a Function of Genotype.* Irrespective of sex and treatment, G2019S animals displayed greater ambulatory width between the hind paws compared to WT animals. Data are expressed as mean $\pm$ SEM. * $p < .01$ simple comparisons of treatment, † $p < .05$ main effect of genotype. LPS+PQ: lipopolysaccharide + paraquat; WT: mice that are wild-type for LRRK2; G2019S: mice that bear the G2019S mutation in LRRK2.

Rotarod. A three-way ANOVA was conducted to assess the effect of sex, genotype, and treatment on the animals' ability to stay on an accelerating rotating rod. Animals that displayed visible signs of sickness were, once again, removed from Rotarod analyses so as to avoid sickness interference effects on motor coordination and balance. Sex and genotype did not significantly influence the time spent on the accelerating rotating rod. However, a significant main effect was apparent for treatment, $F(1, 74) = 14.70$, $p = .0003$, such that mice that received the LPS+PQ treatment ($M = 261.83$, $SE = 13.90$) spent significantly less time on the accelerating rotating rod compared to mice that received the control saline ($M = 292.60$, $SE = 5.32$) (Figure 8). This finding suggests that the LPS+PQ treatment regimen produces significant deficits in motor coordination and balance.

Figure 8. *Time Spent on Rotarod Varied as a Function of Treatment.* Irrespective of sex and genotype, mice exposed to the combined LPS+PQ treatment regimen spent significantly less time on an accelerating rotating rod compared to mice treated with saline. Data are expressed as mean $\pm$ SEM. $*p < .05$. LPS+PQ: lipopolysaccharide + paraquat; WT: mice that are wild-type for LRRK2; G2019S: mice that bear the G2019S mutation in LRRK2.

Immunohistochemical Analyses

TH quantification. A three-way ANOVA was conducted to assess the effect of sex, genotype, and treatment on the amount of TH-positive cells within the SNc. In the absence of a three-way interaction, a significant Sex by Treatment interaction was apparent regarding the number of TH-positive cells within the SNc, $F(1, 30) = 10.99$, $p = .002$. Subsequent comparisons revealed that LPS+PQ treated females, irrespective of genotype, had significantly less TH-positive cells within the SNc than either of female saline controls or males treated with LPS+PQ ($p < .05$) (Figure 7). Further, WT male mice displayed a modest but significant reduction of TH-positive cells following LPS+PQ treatment, relative to the saline treated counterparts ($p < .05$). The toxicants were unable to induce

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significant TH-positive cell loss in the G2019S males. These findings demonstrate that the combined LPS+PQ treatment regimen produced a more robust loss of TH-positive neurons in female mice.

Figure 9. *The Number of TH-Positive SNc Neurons Varied as a Function of a Sex by Treatment*

Interaction. LPS+PQ exposure caused a significantly greater reduction in total TH-positive cells within the SNc of female mice when compared to male mice, irrespective of genotype. Data are expressed as mean $\pm$ SEM. * $p < .05$, ** $p < .01$ simple comparisons of treatment. LPS+PQ: lipopolysaccharide + paraquat; WT: mice that are wild-type for LRRK2; G2019S: mice that bear the G2019S mutation in LRRK2.

CD68-quantification. The quantification of CD68 within the SNc failed to reveal any difference in microglial reactivity within this brain region as a function of sex, genotype, or toxicant treatment. These findings suggest that the inflammatory priming of microglial activity known to occur two days following a peripheral LPS exposure (Purisai et al., 2007) likely subsides and is non-existent by three weeks following the treatment.

Discussion

Several lines of evidence suggest that Parkinson's disease (PD) is a multifactorial condition arising from exposure to a constellation of environmental insults, in conjunction with genetic vulnerabilities, sex differences, and pathological aging (Betarbet et al., 2006; Kelada et al., 2006). Evidence supporting the multifactorial nature of PD etiopathology is highlighted by the fact that either risk factor alone is typically not sufficient to cause the disease. Epidemiological studies have demonstrated a heightened risk for developing PD among individuals chronically exposed to the herbicide, paraquat (PQ), often in conjunction with exposure to multiple other pesticides and/or environmental toxicants (Liou et al., 1997, Tanner et al., 2009). Although these findings have informed the development of animal PD models using such toxicants, even at high doses, PQ treatment in animals does not recapitulate all the clinical features of PD. Indeed, motor and non-motor symptoms are not always present following PQ treatment in rodents and the degree of DA neuronal degeneration is somewhat variable (Brooks et al., 1999; Mangano & Hayley, 2009; Mitra et al., 2011; Purisai et al., 2007). Similarly, while systemic exposure to the inflammatory inducer, lipopolysaccharide (LPS), has been shown to augment the neurodegenerative impact of PQ (Purisai et al., 2007), its ability to induce degeneration of the nigrostriatal DAergic pathway when administered on its own is contentious and often exceedingly high doses are required. Indeed, to date, all PD toxin models (e.g. LPS+PQ, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and 6-hydroxydopamine (6-OHDA) models) fail to recapitulate the complete clinical course of PD, suggesting that additional unknown factors are at play.

At the same time, it is apparent that genetics on its own does not solely precipitate the disease in the majority of cases. Notably, the prevalent G2019S mutation in the leucine-rich repeat kinase 2 (LRRK2) gene has a relatively low clinical penetrance of 28% in 59 year-old individuals (Healey et al., 2008b). Although the penetrance of G2019S increases with age to 51% and 74% at 69 and 79 years of

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age, respectively (Healey et al., 2008b), a substantial number of individuals are still unaffected. Thus, while age represents an essential risk factor, additional environmental components are likely necessary to precipitate the PD pathology. Correspondingly, aged animals overexpressing the G2019S mutation sparingly exhibit modest clinical symptoms (Lee et al., 2015; Li et al., 2010). These data raise the possibility that the G2019S mutation may act as a vulnerability factor that could enhance the neurotoxic actions of environmental risk factors. Accordingly, a recent report found that when G2019S transgenic mice were treated with lactacystin, a proteasome inhibitor, they displayed greatly enhanced nigrostriatal dopaminergic degeneration and greater decline in striatal DA content compared to G2019S-alone and wild-type mice (Xiao et al., 2015). Moreover, this effect was more pronounced at 12 months compared to 5 months, indicating increased pathology with the passage of time and/or advancing age (Xiao et al., 2015).

In addition to aging, environmental factors, and genetic vulnerabilities, sex has proven to be a prevailing risk factor for the development of this disease. Indeed, at all ages and among all nationalities the male sex represents a significant risk factor, with a male to female rate of incidence varying from 1.37–3.7 (Gillies et al., 2014). Although it is uncertain precisely what accounts for the discrepancy between the sexes, evidence suggests that female gonadal steroids, particularly estrogens, have potent anti-inflammatory and anti-oxidative CNS effects that are not necessarily mimicked by male sex hormones (Bourque et al., 2009). The protective effects of estrogen are apparent when estrogen levels are low at menses or diminished with a bilateral ovariectomy resulting in a worsening of PD symptom severity (Quinn & Morsden, 1986) or increased risk for developing PD (Rocca et al., 2008), respectively. Moreover, estrogen replacement therapy has been shown to relieve PD symptoms when given at early stages in the disease (Benedetti et al., 2001) and can even decrease the risk of developing PD (Liu & Dluzen, 2007). Correspondingly, it has been shown that male rodents exposed to DA toxins, such as 6-OHDA and MPTP, tend to display greater reductions in striatal concentrations of DA than do

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females (Liu & Dluzen, 2007). Similarly, it has been found that chronic PQ treatment in female mice is unable to induce significant striatal DA loss when compared to saline controls (Litteljohn et al., 2011). Together, these findings suggest a resiliency in females to the neurotoxic actions of PQ. However, current knowledge on how biological sex influences actual DAergic neuronal loss within the substantia nigra pars compacta (SNc), as induced by toxicants, remains largely unknown. Similarly, sexual dimorphisms in the transgenic G2019S-LRRK2 animal models remain to be determined.

The primary objective of this thesis research was to assess potential additive or synergistic effects of LRRK2 genetic anomalies and environmental risk factors as they pertain to motor behaviour, nigrostriatal DAergic degeneration and indices of neuroinflammation. Moreover, due to the fact that sexual dimorphisms are often overlooked and that the male sex represents a significant risk factor in the development of PD, basic sex differences were also determined. To this end, male and female C57BL/6 mice bearing either G2019S- or WT-LRRK2 genotype received either a combinatory LPS+PQ treatment regime or control saline treatment.

Motor Deficits

Although central LPS infusion followed by peripheral PQ exposure has reported to induce pronounced motor deficits (Mangano & Hayley, 2009), the impact of systemic LPS administration together with PQ exposure on motor behaviour has yet to be addressed. Notably, we previously found that mice centrally infused with LPS followed by i.p. PQ treatment displayed profound splaying of the hind limbs and slow shuffling gait, characterized by a start-stop pattern of movement and dragging of hind limbs when placed in an open field (Mangano & Hayley, 2009). Moreover, these deficits in motor activity exceeded ratings of motor impairment in animals that received LPS or PQ alone (Mangano & Hayley, 2009). Consistent with these findings, we presently found that peripheral LPS treatment

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followed by PQ exposure also caused significant motor deficits as assessed by three different behavioural tests.

Throughout the experimental testing session, mice exposed to the combinatory systemic LPS+PQ treatment regime displayed significantly reduced spontaneous home-cage locomotor activity. Importantly, all home-cage measurements occurred greater than 24 hours following their last PQ treatment so as to avoid potential acute toxic effects of the herbicide. Additionally, to avoid sickness interference effects, animals were assessed for sickness behaviour and were removed from the behavioural assessment if sickness was observed at the time of motor testing. By the fifth PQ injection, mice in the LPS+PQ condition also displayed significant deficits in motor coordination and balance, as measured using a Rotarod apparatus. Once again, animals displaying sickness behaviours were removed from the behavioural assessment. These deficits in motor activity and balance/coordination occurred irrespective of sex or genotype, indicating the absence of any sexual dimorphisms or genetic influence. Interestingly, however, a sex by treatment effect did emerge when gait was assessed using the computer-assisted Catwalk apparatus.

A clear sexual dimorphism was found with regards to gait, such that LPS+PQ treated females responded with enhanced spreading of the front paws as they ambulated, whereas males had a narrowed width in front paw distance. The enhanced spreading of the paws is thought to be an indication of impaired gait. Indeed, previous findings using Catwalk software have reported increases in the average ambulatory width in paws following MPTP exposure in C57BL/6 mice (Wang et al., 2012). Consistently, Mangano and Hayley (2009) reported profound splaying of the limbs in response to peripheral PQ exposure following central LPS priming of the SNc. Conversely, the narrowing of width between the forelimbs observed in males is also presumably a challenging mode of ambulation but it is unclear if this reflects a true impairment in gait.

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Contrary to our initial hypothesis, the interactive effect of sex and treatment on ambulatory width distance between the paws occurred irrespective of genotype. Intriguingly, however, a main effect of genotype was found, such that G2019S mice displayed significantly greater width between both front and hind paws compared to WT mice. Since a splay in the paws is argued to be indicative of a deficit in gait performance these data suggest that mice overexpressing the G2019S mutation have a basal impairment in gait and this was detectable by 11 weeks of age. Current descriptions of the motor performance in mice bearing the G2019S-LRRK2 mutation are inconsistent with reports ranging from hypoactivity (Chen et al., 2012) to hyperactivity (Lango et al., 2014), to no change in motor behaviour (Li et al., 2010). Further, any such detectable effects were modest and only apparent in aged mice (Chen et al., 2012; Lango et al., 2014). The inconsistent effects of the G2019S mutation on motor performance may be due, in part, to the sensitivity of the motor tests applied. Indeed, previous studies have largely involved blindly rating an animal's gait performance in an open field test. The present, computer-assisted Catwalk software may provide a more sensitive and unbiased means of assessing gait performance. That being said, any behavioural effects of the G2019S mutation do appear to be subtle and it remains to be determined if they are biologically meaningful.

Nigrostriatal Dopaminergic Neurodegeneration

Contrary to our expectations, no synergistic or additive effects were observed with regards to any interactive effects of the G2019S mutation and LPS+PQ exposure. Instead, comparable, if not greater degenerative effects were observed among the WT animals. Specifically, WT and G2019S females displayed similar DAergic neuronal loss, corresponding to an approximate 40% and 33% reduction in the amount of TH-positive neurons in the SNc, respectively. G2019S males, on the other hand, displayed no degeneration, despite WT males having a modest (~15%) loss in SNc TH-positive

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neurons. Thus, by these reports, not only was the G2019S mutation unable to enhance or facilitate the neurotoxicant effects as was postulated, it may have been serving neuroprotective effects.

Previous reports indicate that the G2019S mutation may induce modest disruptions in nigrostriatal DAergic signaling, DAergic neuronal survival, and behaviour, but such effects were restricted to aged G2019S mice (Chen et al., 2012; Lee et al., 2015; Li et al., 2010). Moreover, these disruptions were found to be progressive, with a worsening in symptoms occurring with advancing age (i.e. between 12 and 16 months) (Chen et al., 2012). Consistently, in a recent report that looked at the interactive actions of the G2019S mutation and the proteasomal inhibitor, lactacystin, synergistic effects in DAergic cell loss were, similarly, observed in aged mice (Xiao et al., 2015). Thus when considering these previous reports and the absence of a genotype effect in the current study, it appears that age is an indispensable factor required to bring forth the G2019S relevant pathology. Hence, a major caveat of the present study is that relatively young mice were utilized.

To our surprise, G2019S males displayed no loss of TH-positive neurons in response to the LPS+PQ treatment, despite the fact that WT males displayed modest degenerative effects. Thus, if anything, the G2019S mutation may provide protective effects in males at early ages, whereas the opposite might be true at more advanced ages (Chen et al., 2012; Lee et al., 2015; Li et al., 2010). Presently, the role of the G2019S mutation in young animals remains to be fully fleshed out. Considering the current data, it may be that the G2019S mutation promotes the development of compensatory mechanisms or age-dependent shifts in LRRK2 and other signaling pathways, that account for the observed resiliency in young mice and vulnerability in old mice. One noteworthy pathway that may be underlying this effect is the mitogen-activated protein kinase (MAPK) signaling cascade.

It has been shown that the kinase domain of LRRK2, for which the G2019S mutation is found, closely resembles mixed lineage kinases (MLKs) (Mata et al., 2006). The MLKs have been widely

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described for their roles in activating neuronal death signaling pathways by phosphorylating and activating downstream MAPK/c-Jun-N-terminal kinases (JNKs) that mediate neuronal death (Wang et al., 2004). More recently, however, MLKs in the opposing role of cell proliferation and survival through the activation of the MAPK/extracellular signal regulated kinase (ERK) pathway, is beginning to be appreciated (Chadee & Kyriakis, 2004). When considering the dual roles of the MAPK kinase families, it would be interesting to assess how the enhanced kinase activity, induced by G2019S, changes overtime. Indeed, it is conceivable that the heightened kinase activity of the G2019S mutation selectively induces cell survival pathways in young mice, while inducing cell death in aged animals. Further in depth investigations into the mechanisms of LRRK2-MAPK signaling at different ages would be required to delineate these age-dependent effects.

Moreover, the role of MAPK kinases in G2019S related pathology becomes an even more intriguing avenue of study when sex differences and environmental toxins are considered. Previous research has shown that 17β estradiol acts on MAPK/ERK pathways to inhibit the action of proapoptotic protein, glycogen synthase kinase 3 beta (GSK3 β) (Bourque et al., 2009). Interestingly, DA toxins, such as MPTP and 6-OHDA, inhibit GSK3 β phosphorylation, therefore allowing GSK3 β to remain active to potentially contribute to the death of DAergic striatal neurons (Bourque et al., 2009). In the context of the present model, MAPK signaling may provide a molecular account of LRRK2 genotype, sex, and toxicant interactive effects on cell survival and death, and would thus, be an interesting avenue of research to consider in future studies.

Paralleling the gait changes, the number of SNc TH-positive neurons varied as a function of sex and LPS+PQ treatment. Indeed, once again a similar pattern emerged, such that females were affected to a much greater extent than males. Specifically, LPS+PQ treatment induced a preferentially greater loss of SNc TH-positive neurons in female mice. Based on epidemiological data reporting female resiliency to PD pathology (Gillies et al., 2014) along with reports that female mice are more resistant

than males to CNS inflammatory and oxidative insults (Bourque et al., 2009), the observed findings were completely unexpected.

Our own previous research indicated that chronic PQ exposure, similar to the present treatment regime, reduced striatal DA content in male mice (Li et al., 2005; Litteljohn et al., 2009), but had no such effect in female mice (Litteljohn et al., 2011). These findings are consistent with previous reports showing that exposure to other DA toxins, such as 6-OHDA and MPTP, cause greater reductions in striatal DA content in male rodents when compared to females (Liu & Dluzen, 2007; Smith & Dahodwala, 2014). While the mechanisms responsible for such differences remain unclear, accumulating data on the sexual dimorphisms in DA toxicity have focused on gonadal sex hormones, notably 17 β estradiol, as a prevailing factor underlying these sex differences. In a recent meta-analysis, a total of thirty-one studies were shown to demonstrate that estradiol has neuroprotective benefits for DA neurons (Smith & Dahodwala, 2014). Notably, ovariectomizing female rodents (i.e. removing most peripheral source of estradiol) has been reported to enhance striatal DA depletion in response to 6-OHDA (McArthur et al., 2007; Murray et al., 2003) or MPTP (Dluzen et al., 1996) treatment. This striatal DA reduction is restored to normal levels when physiological estradiol levels are replenished (Dluzen et al., 1996; McArthur et al., 2007; Murray et al., 2003), thus advocating estradiol as having a true neuroprotective benefit.

The posited neuroprotective effects of estradiol are supported by several lines of evidence. For example, treatment with estradiol or events associated with high estrogen levels have been shown to enhance DA release by modulating neuronal terminal excitability. Specifically, both basal and amphetamine-induced striatal DA release are found to be greater in rodents during the proestrus phase of the cycle, when estrogen levels are high, as opposed to diestrus, when estrogen levels are low (Becker, 1999). Conversely, ovariectomizing animals prior to post-pubertal surges in sex hormones

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attenuated amphetamine-induced striatal DA release, while once again, estradiol replacement rapidly enhanced DA release (Becker, 1999).

Previous research has shown that estrogen provokes the down-regulation of D2 autoreceptors located on DA terminals, thereby reducing their inhibitory actions on DA release (Becker, 1999). Simultaneously, estrogen inhibits Ca^{++} influx into striatal GABAergic neurons, so as to attenuate their inhibitory signals (Becker, 1999). Thus, estrogen could enhance DAergic neuronal excitability by acting on membrane receptors located in striatal GABAergic neurons and on DA terminals (Becker, 1999). Intriguingly, however, the pro-dopaminergic and beneficial effects of estradiol do not appear to be recapitulated in males (Becker, 1999; McArthur et al., 2007; McDermott et al., 1994). These findings raise the possibility that there are sexual dimorphic effects in the organization and function of the nigrostriatal system that renders males less sensitive to the beneficial effects of estrogen.

When considering sexual dimorphic effects independent of hormonal status, males appear to have more robust dopaminergic activity than females. Notably, when hormone levels are equalized, by gonadectomizing mice, males display significantly greater basal and potassium-induced striatal DA release when compared to females (McDermott et al., 1994). Furthermore, irrespective of hormonal status, male mice and rats have approximately 20% more TH-immunoreactive neurons in the SNc than females (Dewing et al., 2006; McArthur et al., 2007). These findings demonstrate that in the absence of sex hormones, females display greater reductions in DAergic activity than males, implying greater significance on the presence of sex hormones in females for normal DAergic functioning to occur. In other words, males appear to have inherent organizational features that enable more robust DAergic activity relative to females, despite sex hormone levels being depleted. An important factor proposed to account for these sexual dimorphisms in the organization and function of the nigrostriatal system is the Y-chromosome-linked male-determining SRY gene (Smith & Dahodwala, 2014). In male mice, SRY is expressed in the substantia nigra and has been found to co-localize with TH-positive neurons (Czech et

al., 2012; Dewing, 2006). Given that a rise in SRY expression precedes a rise in TH producing DA neurons, it is tempting to speculate that the SRY gene may play a role in mediating DAergic cell development (Czech et al., 2012). In support of this hypothesis, SRY knockdown results in decreased TH expression (Czech et al., 2012; Dewing, 2006), while SRY overexpression results in heightened TH levels (Tao et al., 2011). Moreover, SRY-overexpressing cells also display increased resistance to hydrogen peroxide-induced cytotoxicity (Tao et al., 2011). Together, these data propose a pro-dopaminergic and neuroprotective mechanism in males that is distinct from females and from post-pubertal hormonal levels.

Thus, when considering the aforementioned data, it has been postulated that males have greater inherent neuroprotective features than females, part of which is presumably built-in by the presence of the SRY gene (Smith & Dahodwala, 2014). Without the presence of the SRY gene that is responsible for the initiation of male sex determination, the default phenotype is female; a system that develops in anticipation of later estrogen exposure. Thus, if the female central nervous system relies on later exposure to estrogens, it is conceivable that their absence can produce deficits that supersede those observed in males.

In the current thesis it was hypothesized that females would be less vulnerable to LPS+PQ induced DAergic degeneration and motor deficits on the assumption that females have higher circulating levels of estradiol (which has established beneficial effects on DA neurons). However, a major caveat of the present work is that cyclic estrogen levels were not directly assessed or controlled. Consequently, the neuroprotective effects of estrogen could have been diluted owing to randomized stages of the cycle and consequently varying estrogen levels. Moreover, it is also important to consider that the mice used in the study were relatively young, aged 8 to 10 weeks. At this age female mice have only been cycling for approximately 2 to 4 weeks. Perhaps the beneficial effects of estrogen are accumulative and neuroprotective effects only emerge with the passage of time following initial

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hormonal exposure and/or chronic estrogen receptor activation. To this end, a study assessing over 2000 patients and controls found that women who had undergone a unilateral oophorectomy (i.e. removing a large source of peripheral estrogen) had an increased risk for developing PD only when the surgery was performed before the age of 42 (Rocca et al., 2000). Oophorectomies performed after the age of 42 did not pose the same heightened risk for developing PD, suggesting that duration of lifetime estrogen exposure impacts later PD development (Rocca et al., 2000). In a further case control study, PD was associated with lower cumulative lifetime estrogen exposure, or a shorter fertile lifespan (RS-P et al., 2009; Ragonese et al., 2004). Finally, in a cross-sectional study of women with PD, increased length of endogenous estrogen exposure was associated with older age of disease onset and less severe motor impairments (Cereda et al., 2013). Taken together, these data suggest that longer estrogen exposure during a female's lifetime may decrease the risk of PD. Hence, the greater DAergic degeneration we observed in females may be attributable to low lifetime estrogen exposure or alternatively to potentially diluted levels of cyclic estrogen.

Lastly, when interpreting the current data it is important to consider that the present research involved the peripheral administration of toxins. Prior to now, research reporting sex differences and female resiliency have largely been described in centrally administered toxin models (i.e. 6-OHDA) (McArthur et al., 2007; Murray et al., 2003). Interestingly, the female resiliency observed in these models has not been consistently replicated in studies that apply DA toxins peripherally (i.e. MPTP) (Sedalis et al., 2000). Now, while this inconsistency could be attributable to differences in the toxins themselves, the route of toxin administration may be playing a vital role in how sexual dimorphic effects are expressed in the brain. Of important consideration is the fact that peripherally administered toxins (i.e. MPTP and PQ) are subjected to peripheral sex differences in the uptake and metabolism of these compounds. Indeed, sex-based differences in hepatic drug-metabolizing enzymes and renal clearance have been widely reported in the literature (Waman & Holloway, 2009). Notably, it has been

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found that lower body weight can contribute to lower renal clearance of drugs that are eliminated by the kidneys (Gandhi, 2004). Considering the female mice used in the current research had lower body weight than the males and since PQ is excreted primarily by the kidneys, the rate of toxin clearance may have been lower among the females. As such, the female mice may have been subjected to a longer duration of toxin exposure as a consequence of low renal clearance rate, resulting in greater neurotoxic effects.

Nigrostriatal Neuroinflammation

Microglial cells have been implicated in the pathogenesis of neurodegeneration in PD and in experimental models of PD (Block et al., 2007). Previous research has shown that peripheral exposure to LPS followed by peripheral treatment with PQ two days later, augments microglial activation in the SNc seven days later (Purisai et al., 2007). In the current study, however, peripheral LPS treatment followed by PQ did not result in significant SNc microglial activation as measured by CD68 immunoreactivity three weeks later. The absence of observable microglial reactivity may be attributable to the time of sacrifice relative to the LPS exposure. Previous research has shown that the increased microglia activity that is observed five days following a single peripheral treatment with LPS in C57BL/6J mice subsides 19 days post treatment (Bodea et al., 2014). Thus, while LPS priming of microglial reactivity likely contributes to the initial degenerative effects that follow with subsequent PQ exposure, their activation state returns to baseline three weeks later.

Conclusions and Future Directions

The present thesis research calls into question the nature of G2019S related pathology. Contrary to expectations, the G2019S mutation did not appear to predispose animals to greater neurotoxic or behavioural consequences following exposure to environmental neurotoxins. While the G2019S

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mutation appeared to basally influence gait, evidenced by overall increased splaying of the limbs when compared to WT controls, the mutation did not influence sensitivity to the toxicant challenge. Furthermore, G2019S mice displayed comparable, if not less, degrees of DAergic degeneration following systemic LPS+PQ exposure compared to that of WT mice. Prior to now, all reports of G2019S related nigrostriatal DAergic damage have been described in aged mice (Chen et al., 2012; Lee et al., 2015; Li et al., 2010). Indeed, the synergistic effects of the G2019S mutation and proteasomal inhibition on DAergic neuronal loss were reported in aged animals (i.e. 12 months) (Xiao et al., 2015). Thus, it appears that the G2019S mutation may confer age-associated susceptibility to toxin-induced nigrostriatal DAergic degeneration. When considering these previous reports with our current findings, it becomes evident that age may be an indispensable feature required to precipitate G2019S related pathology.

Moreover, G2019S males displayed no significant degeneration in response to the toxicant, despite WT males displaying modest degenerative effects. Thus, not only was the G2019S mutation insufficient at facilitating neurotoxin-induced pathology, it may have been providing neuroprotective benefits at this age. Taken together, our data implies a potential novel neuroprotective role for G2019S in young animals, whereas the opposite appears to be true at more advanced ages (Chen et al., 2012; Lee et al., 2015; Li et al., 2010; Xiao et al., 2015). To this end, future research aims to further delineate the role of age in G2019S related pathology by investigating the interactive effects of the G2019S mutation and LPS+PQ toxicant challenge at multiple ages. Moreover, future goals of research involve addressing potential age-dependent compensatory mechanisms or shifts in G2019S signaling. Notably, we wish to examine the interactive effect of age, LRRK2 genotype, and neurotoxicants on MAPK signaling. Since LRRK2 kinase activity involves the downstream activation of MAPK kinases (Mata et al., 2006), and since the activation of different MAPK subfamilies can lead to opposing actions in terms of cell

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survivability (Chadee & Kyriakis, 2004; Wang et al., 2004), studying these enzyme pathways are of particular interest.

Lastly, extensive research is required to fully characterize the nature of sexual dimorphisms in neurodegeneration and behavioural deficits elicited by exposure to LPS+PQ. Inconsistent with previous research and claims of female resiliency to DA neurotoxicity, our data shows that female mice displayed greater splaying of the hind limbs (i.e. deficit in gait) and greater degrees of DAergic cell loss when compared to males. Future directions of research aim to determine how the presence or absence of steroidal hormones, notably estradiol, influences neuronal survival. This can be achieved by gonadectomizing the mice, removing most of the peripheral sources of steroidal sex hormones, and supplementing estradiol (or testosterone) via mini-pump infusions or subcutaneous injections. Due to inherent sexual dimorphisms in nigrostriatal functioning, independent of post-pubertal hormonal status, it would be intriguing to see how the pro-dopaminergic effects of the male-determining SRY gene serve to protect males.

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