A role for LRRK2 and neuroinflammatory processes in multi-hit toxicant models of Parkinson's disease

Christopher Alan Rudyk

A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in partial fulfillment of the requirements for completion of the degree of

Doctor of Philosophy
in
Neuroscience

Carleton University
Ottawa, Ontario

Abstract

Parkinson's disease (PD) is characterized by the loss of dopaminergic neurons in the substantia nigra (SNc) leading to a range of motor behavioral deficits. In addition to the cardinal motor features of the disease, non-motor behaviors are also evident in many cases. Although it has been suggested that genetic mutations represent a cause or risk factor for the disease, there is evidence to suggest that PD arises as a result of the interaction between multiple factors. In the current dissertation, one overarching theme we were interested in was how the behavioral and neurochemical effects of the PD relevant herbicide paraquat (a chemical stressor) might be impacted when combined with different stressors including chronic psychological stress, immune stress, or age induced alterations. Furthermore, we were also highly interested in providing further understanding regarding the role of neuroinflammatory processes (as occurs in PD) in the paraquat induced death of SNc dopamine neurons. In these instances, we focused on the inflammatory regulatory gene (and number one gene implicated in PD), leucine rich repeat kinase 2 (LRRK2). Accordingly, in our study combining paraquat exposure with a chronic unpredictable stress regimen, we found that chronic psychological stressor exposure did not influence the degeneration of midbrain dopamine neurons or accompanying microglia activation induced by the toxin; however, it did influence motor coordination. Conversely, exposure of the pesticide in combination with the inflammatory agent lipopolysaccharide (LPS) augmented SNc cell loss. In these studies using LPS, we found that knocking out LRRK2 protected against the loss of midbrain dopamine

neurons and behavioral deficits, induced by LPS priming followed by paraquat exposure. In fact, knocking out LRRK2 altered the pro-inflammatory microglia phenotype that is typically induced by LPS exposure. Likewise, LRRK2 deficiency protected against the paraquat induced peripheral and central toxic effects in mice older than what we typically use in our models. Taken together, the present dissertation supports the hypothesis that the interaction between different stressors can impact behavioral and biological outcomes relevant for PD, and LRRK2 is important for the toxic effects of paraquat, and LPS priming with later paraquat exposure. The data presented herein may also provide important implications for the development of treatment strategies that target inflammatory processes in PD, to halt or slow the progression of the disease.

This thesis is based on the following three manuscripts referred to in the text by their corresponding chapter numbers.

Chapter 1: Rudyk CA, Dwyer Z, Farmer K, McNeill J, Wahbeh F, Prowse N, Hayley S. Chronic unpredictable stress differentially influenced impact of paraquat upon behavioral and neuronal outcomes. *In Review.*

Chapter 2: Rudyk CA, Dwyer Z, Thompson A, Farmer K, Fortin T, Derkesen A, Hayley S. Leucine rich repeat kinase-2 (LRRK2) modulates microglial phenotype and dopaminergic neurodegeneration.

Chapter 3: Rudyk CA, Dwyer Z, Hayley S. Age-dependent paraquat toxicity: Effects of LRRK2 KO.

Other works by Christopher Rudyk

Published

(1) Simard S, Coppola G, **Rudyk CA,** Hayley S, Salmaso N (2018). Towards a neuroplasticity hypothesis of depression: astroglia as key mediators. Neuropsychopharmacology.

DOI: 10.1038/s41386-018-0105-x
PMID: 29907879

(2) Boutin R, Dwyer Z, Farmer K, **Rudyk CA**, Forbes M, Hayley S (2018). Perinatal antibiotic exposure alters composition of gut microbiota and promotes allergic sensitization to peanut antigen. Allergy, Asthma & Clinical Immunology.

DOI: TBD
PMID: TBD

(3) Litteljohn D, **Rudyk CA**, Dwyer Z, Farmer K**,** Fortin T, Hayley S (2017). The impact of murine LRRK2 G2019S transgene overexpression on acute responses to inflammatory challenge. Brain, Behaviour, and Immunity.

DOI: 10.1016/j.bbi.2017.09.002
PMID: 28893563

(4) **Rudyk CA**, McNeill J, Prowse N, Dwyer Z, Farmer K, Litteljohn D, Caldwell W, Hayley S (2017). Age and chronicity of administration dramatically influenced the impact of low dose paraquat exposure on behavior and hypothalamic-pituitary-adrenal activity. Frontiers in Aging Neuroscience.

DOI: 10.3389/fnagi.2017.00222.
PMID: 28769783

(5) Litteljohn D, **Rudyk C**, Razmjou S, Dwyer Z, Syed S, Hayley S (2017). Individual and interactive sex-specific effects of acute restraint and systemic IFN-γ treatment on neurochemistry. Neurochemistry International.

DOI: 10.3389/fnagi.2017.00222
PMID: 27876634

(6) Razmjou S, Litteljohn D, **Rudyk C**, Syed S, Clarke M, Pentz R, Dwyer Z, Hayley S (2016). The interactive effects of ketamine and magnesium upon depressive-like pathology. Neuropsychiatric Disease and Treatment.

DOI: 10.2147/NDT.S111131
PMID: 27660449

(7) **Rudyk CA**, Litteljohn D, Syed S, Dwyer Z, Hayley S (2015). Paraquat and psychological stressor interactions as pertains to Parkinsonian co-morbidity. Neurobiology of Stress.

DOI: 10.1016/j.ynstr.2015.09.001
PMID: PMC4730791

(8) Farmer K, **Rudyk CA**, Prowse N, Hayley S. Hematopoietic cytokines as therapeutic players in early stages of Parkinson's disease. Frontiers in Aging Neuroscience (2015).

DOI: 10.3389/fnagi.2015.00126.
PMID: 26191001

(9) Liu G, Rustom N, Litteljohn D, Bobyn J, **Rudyk C**, Anisman H, Hayley S. Use of induced pluripotent stem cell derived neurons engineered to express BDNF for modulation of stressor related pathology. Frontiers in Cellular Neuroscience (2014).

DOI: 10.3389/fncel.2014.00316
PMID: 25352778

(10) Osborn M, Rustom N, Clarke M, Litteljohn, D, **Rudyk C**, Anisman H, Hayley S. Antidepressant-like effects of erythropoietin: A focus on behavioural and hippocampal processes. PlosOne (2013).

DOI: 10.1371/journal.pone.0072813.
PMID: 24019878

Submitted

(1) Jadavji NM, Murray LK, Emmerson JT, **Rudyk CA**, Hayley S, Smith PD. Paraquat exposure increases oxidative stress within the dorsal striatum of male mice with a genetic deficiency in one-carbon metabolism. Toxicological Sciences (submitted June 2018).

(2) Farmer K, Derksen A, Rowe E, Thompson A, **Rudyk CA**, Prowse NA, Dwyer Z, Fortin T, Abd-Elrahman KS, Ferguson SSG, Hayley S. mGluR5 modulation promotes neurorecovery through mTOR in a Parkinson's model. Annals of Neurology (submitted June 2018).

Acknowledgements

I would like to thank my family, friends, members of the Department, and all of the members of the Hayley lab (past and present) that I have had the pleasure of working with. Without each and every one of you, I would not have had either the emotional support, guidance, and/or expertise needed to complete this degree, which was often very emotionally challenging. Most importantly, I'd like to thank my supervisor, Dr. Shawn Hayley. Dr. Hayley has provided me tremendous guidance as well as an environment that facilitates both learning and growth. Shawn, I am extremely grateful for your patience, support, and belief in me throughout the last 8 years. Thank you.

Table of Contents

List of Figures

General Introduction

CHAPTER 1: Paraquat and chronic stress interactions as it pertains to Parkinson disease motor and co-morbid behavior deficits

CHAPTER 2: Paraquat and chronic stress interactions as it pertains to Parkinson disease motor and co-morbid behavior deficits

CHAPTER 3: Age-dependent paraquat toxicity: Effects of LRRK2 KO

General Discussion

List of Abbreviations

6-OHDA	6-hydroxydopamine
AAR	Alternate Arm Returns
AD	Alzheimer's Disease
AMPA	l-α-amino-3-hydroxy-5-methylisoxazole-4-propionate
ANOVA	Analysis of Variance
BBB	Blood Brain Barrier
BDNF	Brain Derived Neurotrophic Factor
BCA	Bicinchoninic Acid
CA	Calcium
CNS	Central Nervous System
COX-2	Cyclooxygenase-2
CSF	Cerebrospinal Fluid
CX3CR1	Chemokine Receptor 1
CVO	Circumventricular Organ
DAMP	Damage Associated Molecular Pattern
DAT	Dopamine Transporter
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
GSH	Glutathione
GSSTI	Glutathione S Transferase T1
GWAS	Genome Wide Association Studies
HIV	Human Immunodeficiency Virus
HMGB1	High-mobility Group Box 1
HPA	Hypothalamic–Pituitary–Adrenal Axis
IBA1	Ionized Calcium-Binding Adapter Molecule
IFN-γ	Interferon Gamma
IL-1β	Interleukin-1Beta
IL-6	Interleukin 6

iNOS	Inducible Nitric Oxide
JNK	c-Jun N Terminal Kinase
KO	Knockout
LC	Locus Coeruleus
L-DOPA	Levodopa
LPS	Lipopolysaccharide
LRRK2	Leucine Rich Repeat Kinase 2
MMx	Micromax
MAO	Monoamine Oxidase
MPP+	1-methyl-4-phenylpyridinium
MPPP	1-methyl-4-phenyl-4-propionoxypiperidine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Multiple Sclerosis
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NFAT	Nuclear Factor of Activated T cells
NGS	Normal Goat Serum
NRON	Non-Protein Coding RNA
PAMP	Pathogen Associated Molecular Pattern
PB	Phosphate Buffer
PBS	Phosphate Buffer Saline
PD	Parkinson's Disease
PFA	Paraformaldehyde
pGR	Phosphorylated Glucocorticoid Receptor
PQ	Paraquat
PRR	Pattern Recognition Receptor
RIPA	Radio Immuno Precipitation Assay
PVDF	Polyvinylidene Difluoride
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RR	Rotarod
SAB	Spontaneous Alternation Behaviour
SAR	Same Arm Returns
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SOD	Superoxide Dismutase

STAT	Signal Transducer and Activator of Transcription
SNc	Substantia Nigra Pars Compacta
SNPs	Single Nucleotide Polymorphisms
TH	Tyrosine Hydroxylase
TNF-α	Tumor Necrosis Factor Alpha
TLR	Toll Like Receptor
trkB	Tropomyosin-Related Kinase B
trkb.FL	Tropomyosin-Related Kinase B Full Length
trkB.T2	Tropomyosin-Related Kinase B Truncated
VMAT	Vesicular Monoamine Transporter 2
WASP	Wiskott-Aldrich Syndrome Protein
WAVE2	WASP-Family Verproline Homologous Protein-2
WT	Wild Type

General Introduction

Defining features of Parkinson's disease

Parkinson's disease (PD) is the second most common age-related neurodegenerative disorder, estimated to occur in up to 0.2% of the population 60 years of age and rising sharply thereafter, with instances exceeding 4% by age 85 [1], [2]. While the aetiology of PD is unknown, it is thought that sporadic cases of the disease arise due to the interaction between various environmental insults (from psychological to immune to chemical) and genetic factors [3]. Clinically, PD manifests itself as primarily a movement disorder whereby patients display motor control abnormalities including postural instability, slowness of movement, difficulties in gait and balance, and muscle rigidity (i.e. tremors) [1]. Functionally, these motor control abnormalities emerge as a result of the progressive degeneration of dopamine neurons in a region of the brain termed the substantia nigra pars compacta (SNc) [4], [5]. As such, this gradual and progressive neuronal loss results in reduced release of the monoamine in the downstream striatum, the main innervation and termination site for SNc projection neurons [6]. This neurodegenerative disease is typically diagnosed in humans after patients display a marked reduction in these archetypal behavioral features after administration of the dopamine precursor levodopa (L-DOPA), however complete diagnosis is not entirely confirmed until autopsy [7].

Another biological feature known as Lewy Body inclusions are also a defining characteristic evident in sporadic PD cases [8]–[10]. These inclusions are present throughout the brain parenchyma and are found in the soma and dendrites (effectively termed Lewy neurites) of neurons [11]. Primarily comprised of accumulated misfolded protein aggregates,

Lewy Bodies principally encompass α-synuclein, as well as the limited expression of parkin, ubiquitin and others [6], [10]. Finally, the up-regulation of inflammatory factors including elevated regional expression of inflammatory microglia (the brains resident immune cell), pro-inflammatory cytokines, and the infiltration of peripheral immune cells (i.e. CD8+ and CD4+ T cells) which together collectively result in neuroinflammation- represent a third biological hallmark of PD [12]–[14]. In fact, neuroinflammation (principally controlled by microglia) has been suggested to play a fundamental role in the progressive loss of SNc dopamine neurons and represents a crucial feature of PD [15]. However, despite growing support for inflammation in the disease, there is a need to better understand neuroinflammatory processes that underlie SNc neuron death.

In addition to the motor impairment evident in all PD cases, a substantial number of diagnosed patients also display prominent "nonmotor" symptoms, including autonomic and olfactory problems (e.g., sleep disorders, hyposmia), as well as cognitive and psychological disturbances (e.g., anxiety, depression) [14], [16]–[19]. While the dopamine denervation from the striatum to the SNc may give rise to at least some of these co-morbid symptoms (e.g. memory and attention problems), it is likely that multi-neurotransmitter dysfunction in brain regions important for autonomic, emotional, and psychological functioning (e.g., locus coeruleus, prefrontal cortex, hippocampus) are important in this regard (perhaps stemming from parallel inflammatory and neurodegenerative processes) [1], [18], [19]. It has even been suggested that dysfunction in many of these non-motor systems manifest before the onset of those controlling motor processes and thus a PD diagnosis [1], [18], [19]. For instance,

brainstem structures highly implicated in cognitive and neuropsychiatric behaviours including the noradrenergic locus coeruleus, and serotonergic raphe nuclei are believed to be affected prior to the nigrostriatal pathway, and as such may give reason as to why these disturbances occur *a priori* to clinical onset of the disease [20]. As such, combining non-motor behavioural characteristics in conjunction with other potential early biomarkers may help aide in a clinical PD diagnosis [21], [22].

***General thesis statement*:**

The present thesis is underpinned by two major themes: 1. Characterizing how differing stressors (from psychological to immune to aging) can independently or interactively influence the impact of a chemical toxicant (and stressor), paraquat, to promote biological and behavioural features of PD in mice and 2. Better understanding the role of neuroinflammatory processes in SNc neuronal death (as occurs in PD), with a particular focus on the inflammatory gene, *leucine rich repeat kinase 2* (LRRK2). Upon the background of these two themes, we not only focus upon the characteristic motor features of PD, but also to some degree assess the often overlooked but very important co-morbid non-motor (e.g. cognitive, depression, anxiety) features of the disease. In the sections that follow, we will first outline the evidence for how various environmental stressors (e.g. psychological stress, pesticide exposure) may contribute to the development and evolution of PD. Thereafter, we will discuss the neuroinflammatory aspects of PD and how these might give rise to the motor features of the disease. Finally, we will describe the evidence implicating LRRK2 in PD and introduce why this factor might be a general inflammatory regulator controlling the many aspects of the disease, and as such may be important in paraquat induced toxicity.

Environmental factors and PD

Growing support has been centered around the a multi-hit hypothesis for PD which suggests that onset and progression of the disease likely involves the collective contribution of a spectrum of stressors (from psychological, to immune, to chemical) over time, which in some cases may interact with genetic factors/vulnerabilities to result in a PD diagnosis [23]. While mutations in genes including α-*synuclein* (SNCA), LRRK2, *parkin* (PARK2), *PINK1*, *deglycase* (DJ-1) have been implicated in the manifestation and progression of PD [24], familial related PD only accounts for 5-10% of cases with the vast majority being idiopathic and sporadic in nature [25]. It is thought that the contributing factors responsible for PD likely exist on a spectrum with familial linked forms (e.g. SNCA, LRRK2, DJ-1) that result in early diagnosis at one end and purely environmental impact at the other, leaving the bulk of influence attributed to the interactive effects of genetic vulnerability and environmental influence [26]–[28]. Indeed, the fact that not all highly penetrant genes associated with PD give rise to parkinsonian symptoms and that many polymorphisms have been linked to the disease, strongly suggests the added influence of environmental factors [29]. Additionally, mounting evidence from animal research in the last decade has emerged supporting gene-environment interactions in PD pathophysiology [27], [30]–[32]. In regard to the multi-hit model of PD for example, the first "hit" could be genetic in nature such that gene polymorphisms/vulnerabilities (found in PD related genes including LRRK2, DJ-1) interact with stressors (i.e. chemical, inflammatory, psychological) to additively or synergistically shape the evolution of PD pathology [19].

In regard to sporadic cases of PD, a number of factors have been associated with higher risk of development, including age (the number one risk factor implicated in PD), genetic

polymorphisms, psychological stress, and cumulative exposure to variety of environmental factors [33]. For example, heavy metals like lead and manganese, air pollutants, head trauma, and more recently viral infections have all been implicated as potential contributors to disease pathology [33]. One group of risk factors repeatedly implicated in PD, as made evident by several lines of research and is a major central theme of the current thesis, includes chemical toxicants such as pesticides [26], [27]. While difficult to separate or pin point the exact effects of any single type of stressor, it is likely that some (e.g. chemical pesticides) may selectively target and/or have greater impact on midbrain dopamine neurons, whereas other (e.g. psychological stressors) may be important in imparting neurochemical-related disturbances that result in the neuropsychiatric symptoms associated with the disease, or may even impact disease state only in the presence of another stressor [23], [34]. Nonetheless, the impact of any of these stressor types likely depends on a number of factors including duration and time of exposure, it's temporal exposure in relation to other stressors, and finally the state of the brains microenvironment upon exposure [23], [34].

Pesticide Exposure: Paraquat as a risk factor implicated in PD

The idea that chemicals like pesticides influence PD manifestation came about just over three decades ago when certain individuals synthesizing opioids (1-methyl-4-phenyl-4-propionoxypiperidine- MPPP) for personal use rapidly developed abnormal motor behavioural characteristics reminiscent of PD symptoms after consumption [35]. It turns out the drug was contaminated with the substance, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP),

which is now known to be a highly potent dopaminergic neurotoxin, and is a widely used to model PD in animal studies [36], [37]. MPTP leads to neuronal death via its metabolite, 1-methyl-4-phenylpyridinium (MPP+), by the enzyme monoamine oxidase B (MOAB) present on glial cells [38]. MPP+ is readily taken into dopamine neurons by the dopamine transporter (DAT), whereby it promotes neurotoxicity via interfering with mitochondrial metabolism resulting in production of reactive oxygen species (ROS) [38]. Importantly, MPP+ is similar in chemical structure to the pesticide, paraquat [38]. As such these findings lead to epidemiological data supporting a role for paraquat as a potential environmental risk factor for PD development [28].

Support for pesticide exposure in disease provocation comes from meta-analyses and epidemiological studies primarily conducted on agricultural communities [26]. In fact, several compelling lines of evidence now suggest a role for specific pesticides, such as the non-selective herbicide paraquat, as well as the organic insecticide rotenone, as major risk factors for disease development [27]. These epidemiological findings are supported by a plethora of animal studies (both primate and rodent) demonstrating the ability of these toxins to dose-dependently induce PD-like pathology [39]. Indeed administration of pesticides in rodents, including paraquat and rotenone, induces many of the neuropathological and behavioral features that are characteristic of PD [19], [39]–[41]. For example, our own group (and others) has demonstrated an ability of paraquat to induce the selective loss of dopamine neurons in the SNc and accompanying striatal fiber loss in a dose dependent manner that is reminiscent of what that which occurs in PD [42]–[46]. Likewise, these studies have recapitulated other

behavioural and neuropathological features of the disease including motor behavioural disturbances, oxidative stress, aggregated protein inclusions, and region specific microglia activation which indicates neuroinflammatory processes at play [45], [47]–[49]. As will be discussed later, prolonged microglia mediated neuroinflammatory processes play a key role in the development and progression of PD, and it is likely that one way (amongst others) environmental stressors (such as paraquat) can impact neuronal integrity is through inflammatory driven mechanisms [49], [50]. In a general view, paraquat (and other stressors) can engage neuroinflammatory cascades, which results in augmented responses, that over time (and perhaps in conjunction with genetic vulnerabilities) can damage the susceptible SNc dopamine neurons [49]–[51].

Paraquat itself is a non-selective herbicide used to clear green plant tissue that has been used worldwide for several decades [52]. Amidst the epidemiological findings linking the herbicide to PD, the European Union has banned its use, however many countries have yet to do so [40]. In fact, higher use of the pesticide has been reported in highly populated countries including China and India [40]. Mechanistically, paraquat is able to gain entry into the central nervous system (CNS) via a neutral amino acid transporter present at the tight junction blood brain barrier (BBB) [53], [54]. Upon entry into the brain, the toxin is distributed across areas including the prefrontal cortex, hippocampus, olfactory bulbs, striatum, and SNc [55]. Once in the CNS, paraquat can promote a central inflammatory cascade through microglia activation, as well as gain entry into neurons [49], [51]. Neuronal entry of paraquat requires conversion from its divalent to its monovalent form by redox interaction with the nicotinamide adenine

dinucleotide phosphate (NADPH)-oxidase enzyme present on microglia, whereby it becomes a substrate for DAT, and can thus be taken into dopamine neurons by the transporter [51] (see figure 1). Once in the neuron, it has been shown that the herbicide can be rapidly re-oxidized back to its divalent form by oxygen but this results in the downstream production of a number of ROS including superoxide radicals, hydrogen peroxide, as well as hydroxyl radicals [56]–[60]. Furthermore neuronal entry of the toxin can cause vesicular damage, induce endoplasmic reticulum stress [61], and even react with present α-synuclein resulting in neuronal dysfunction and damage [51], [60], [62]–[66]. Additionally, this potent oxidative stressor can disrupt multiple cellular processes including calcium (CA) homeostasis [67], inhibit different mitochondrial electron transport chain complexes (i.e. complex I and IV), and increase mitochondrial membrane permeability leading to apoptotic factor release [52], [60], [68], [69], all of which have been implicated in PD pathology [69].

Intriguingly, certain genetic mutations have been reported to enhance susceptibly to environmental insults, such as pesticides and hence, might contribute to the more common idiopathic cases of the disease [26]. A recent study for example demonstrated that mutations in DAT can increase risk for developing PD in individuals who experience life-long pesticide exposure, an effect greater than possessing the mutation or being exposed to the pesticide alone [70]. In fact, a number of other mechanisms through which genetic vulnerability enhances susceptibility to environmental insults (i.e. paraquat exposure) resulting in PD have also been suggested. For example, a recent epidemiological study found augmented risk of developing PD in individuals who possessed either the rs1045642 or rs2032582 polymorphisms in the

ABCB1 gene in frequent pesticide sprayers [71]. Importantly, ABCB1 is responsible for encoding P-glycoprotein which acts as a cellular efflux transporter of lipophilic compounds present in the BBB [71], [72]. Both the rs1045642 and rs2032582 polymorphisms decrease expression of P-glycoprotein on epithelial cells lining the BBB which results in increased concentrations of neurotoxic substances (i.e. xenobiotics such as pesticides) penetrating the brain [71]. Moreover, when looking at appliers of the herbicide paraquat, Goldman and colleagues (2012) identified an 11.1 fold increase in risk for PD but only in individuals who also lacked the glutathione S transferase T1 (GSST1) gene, which is primarily responsible for detoxifying xenobiotic compounds [73]. Likewise, individuals possessing polymorphisms in the DAT/SLC6A3 are at increased risk of developing PD when also exposed to environmental insults [70]. It is hypothesized that the DAT/SLC6A3 polymorphisms enhances pesticide entry into neurons [70]. Carriers of the DAT single nucleotide polymorphisms (SNPs), rs2652511 and rs2937639, do indeed have an increase in DAT expression in striatal regions [74] and variations in the transporter can enhance susceptibility to paraquats entry into neurons and subsequent toxicity [26], [75]. A recent animal study for example demonstrated the potential protective effects against the toxin in DAT knock-down mice [75]. These findings support the notion that PD cases likely emerge not from one risk factor alone but from the constellation of environmental insults over the course of one's lifetime that act in elaborate interplay with inherent genetic vulnerabilities

given the discrepancies and inconsistencies arising from both epidemiological studies as well as animal studies recapitulating the pathological effects of paraquat (and rotenone) have been demonstrated. In regard to paraquat exposure, while the majority of animal studies demonstrate ability of the toxin to induce PD associated pathological changes, some have failed to reproduce such effects (Jones et al., 2014). Likewise, whereas the vast majority of epidemiological studies implicate the toxin as a potential risk factor in disease provocation with some identifying an up to three fold risk for development, others have found no increased risk (Jones et al., 2014).

Thus, it appears that while polymorphisms may not cause PD, they do increase susceptibility to the disease by modifying the impact of environmental challenges.

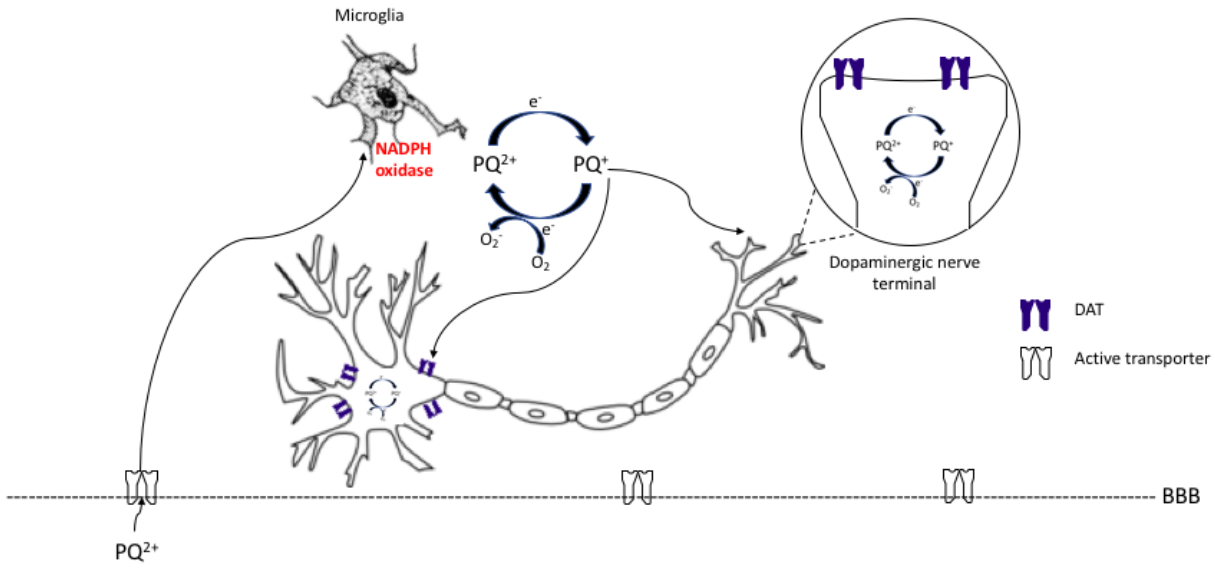

Figure 1: Pathways of paraquat induced neuronal toxicity.

Upon entry into the brain via transport across the blood brain barrier (BBB), the divalent form of paraquat (PQ^{2+}) is converted to its monovalent form (PQ^{+}) by nicotinamide adenine dinucleotide phosphate (NADPH) present on the extracellular membrane of microglia resulting in the production of oxidative radicals. Likewise this reduction allows entry of PQ+ into dopamine neurons via the dopamine transporter (DAT) present on axon terminals and the neuronal cell body where it can then result in the further production of oxidative radicals and neuronal damage.

Psychological stress-environment interactions and PD

Even chronic psychological stressors may also contribute to symptom presentation in PD [76], [77]. In general, exposure to stressful situations triggers a multifaceted neuroendocrine response, which allows the brain to engage in adaptive processes that result in behaviors that enable an individual to respond to situational demands [78]. In response to a stressor, the classic biological reaction involves the release of hormones including corticosteroids (i.e. cortisol) via

hypothalamic-pituitary-adrenal (HPA) axis stimulation, as well as the catecholamine norepinephrine, which can both independently and together profoundly impact brain function [79], [80]. However, chronic activation of this stress system has been linked to a variety of neuropsychiatric diseases including anxiety and depression, as well as cognitive impairment [79].

Notably, chronic stressors have even been proposed to increase the risk of neurodegenerative disorders (including PD) by virtue of their ability to engender neurochemical disturbances [81]–[83], cellular alterations, as well as promote the loss of trophic factors which conventionally act to provide neuronal support [81], [84], [85]. In regard to PD, stress exposure has been shown to influence symptom presentation, as diagnosed patients have reported worsened motor symptoms (i.e. bradykinesia, freezing behavior, and tremor) when under stress [86]. It is thought that stress influence on motor symptom presentation is due, in part, not only to the aforementioned mechanisms, but also to impaired dopamine synthesis and release [87]. Moreover, it has also been suggested that extreme chronic stress can promote the death of SNc dopamine neurons culminating in PD [88]. For instance, animal models of stress have shown that extended chronic restraint (8 hrs /day for 5 days a week up to 16 weeks) can result in the progressive loss of SNc dopamine neurons [88]. In fact, similar to other stressors (i.e. chemical, immune, aging), psychological stressors can also promote/enhance neuroinflammation which can be toxic to neurons (discussed below) [89]–[93]. These neuroinflammatory alterations can also act in conjunction with the release of glucocorticoids (i.e. cortisol or corticosterone in rodents) from chronic HPA axis activation, which can effortlessly penetrate the brain, and at

high levels, also be neurotoxic [94]. Moreover, other peptide hormones released from the active HPA pathway (e.g. corticotropin-releasing hormone; CRH) can also compromise neuron integrity by disrupting BBB permeability, causing the infiltration of a variety of noxious factors [95].

In some cases, the alterations that psychological stressors impart on the brain microenvironment can make it particularly sensitive to additional stressor challenges [96]. In fact, these stress induced alterations, may even promote or enhance cell death when in combination with these other insults [88]. For instance, it has been demonstrated that chronic stress can result in microglia adopting a 'primed' phenotype, which is characterized by having an exaggerated inflammatory response to a subsequent immune stimulus [97], [98]. Indeed chronically stressed animals displayed enhanced microglia reactivity when subsequently exposed to the inflammatory inducing agent lipopolysaccharide (LPS), a result which likely occurs due to stress-induced up-regulation of its receptor (known as toll like receptor-4 (TLR4)) on microglia [99]. Likewise, established animal models of PD using the catecholaminergic neurotoxin 6-hydroxydopamine (6-OHDA) have shown that chronic stress administered both prior to and after 6-OHDA lesioning accelerated and enhanced overall damage to the nigrostriatal system, as well as augmented motor behavioral impairments [76], [100], [101]. These findings are similar to studies using MPTP, who likewise demonstrate exacerbated dopamine SNc neuronal loss in mice also exposed to a chronic mild stress regimen [102], [103]. Moreover, anhedonia (a symptom of depression) was only observed in MPTP treated mice that were also exposed to a chronic stressor, suggesting stress may make mice more vulnerable to

non-motor effects of toxins [102]. In fact, in line with this observation, it is not surprising that recent observations show that major life events can influence the development of depression in PD patients [104]. Whatever the case, these results suggest that the nigrostriatal system may be particularly sensitive to stressor challenges [87], [105], [106], which might have important repercussion for PD symptom emergence and exacerbation [77]. Likewise these results raise the possibility that psychologically relevant stressors experienced prior and during the course of PD could affect both the primary motor symptoms and neurodegenerative processes induced by paraquat [100], [107], [108], as well as certain non-motor or co-morbid neuropsychiatric manifestations.

Neuroinflammation

Over the last 15 years, a major shift has been taken in regard to immune-brain interactions and around the notion that inflammatory processes may contribute to PD development [50]. Only two decades ago the notion that inflammatory processes impact PD was actually met with considerable scrutiny and resistance in part because the brain was highly considered as being immune-privileged, largely protected from peripheral immune events [15], [109]. Today however, accumulating evidence exists supporting a role for neuroinflammatory processes not only being involved in PD, but also virtually all neurological (and even neuropsychiatric) disorders [110]. In the following section, we will give you a general overview of neuroinflammation followed by a brief discussion of the evidence that suggets a role for neuroinflammatory factors in PD neurodegenerative processes.

In general, inflammation typically refers to the classic response a host mounts against an antigen (i.e. a pathogen, damaged tissue, or foreign agent) [83]. Standard clinical features including swelling, redness, pain, and fever, are typically those classic signals that indicate to the host inflammatory processes are at play [111]. The highly coordinated and exquisite inflammatory response involves a wide assortment of circulating immune cells including dendritic cells, macrophages, neutrophils, and leukocytes (aka white blood cells; e.g. natural killer (NK) cells or lymphocytes such as T and B Cells) [112], [113]. In very broad terms, once activated (i.e. via injury or an immune challenge), cells of the immune system can secrete a variety of factors to aide in the host response including the release of nitric oxide (NO), ROS, prostaglandins, and inflammatory messenger proteins known as cytokines (discussed below), which are critical players in adaptive and innate defense processes [83], [114]. Moreover, upon detection of a pathogen or threat, antigen presenting cells (e.g. macrophages, dendritic cells, microglia) identify the insult, and present it to adaptive immune lymphocytes (i.e. T and B cells) via the human leukocyte antigen (HLA) system (or major histocompatibility complex (MHC) in rodents) for self-versus-non-self recognition [113]. Through this HLA mediated presentation of the antigen, the immune system is then primed to mobilize and expand to target and eliminate that specific antigen, as well as gains a memory should that insult ever be presented again, so that it can be promptly dealt with [113].

In a normal healthy state, immune processes in the CNS differ from those in the periphery, in part because of the BBB, and in the classic view of neuroimmunology, the CNS has been typically regarded as immune-privileged, existing independent of peripheral immune influence

[115]. Located in the walls of the blood vessels supplying the CNS, the BBB is composed of an intricate network of cells (i.e. astrocytes, neurons, pericytes, endothelial cells) that form very tight junctions resulting in very low permeability, thus restricting access of peripheral blood borne molecules [115]. As such, the BBB is extremely selective in terms of determining what macromolecules it allows to gain entry, protecting the CNS from blood-borne molecules including infiltration of harmful substances as well as peripheral immune factors, while at the same time regulating the uptake of essential molecules such as nutrients, gases, water, and metabolites [115].

Given its general protection from peripheral immune events, immune processes in the CNS are largely regulated by the immuno-competent microglia (and to a lesser extent astrocytes, and neurons) [50]. Indeed, neuroinflammation (principally involving microglia) generally exists as the CNS's protective response, in which microglia respond to neuronal injury signals (i.e. trophic factors, ATP, adhesion molecules), infection, xenobiotics (i.e. viral infections, drug metabolites, environmental toxins), and other potentially toxic metabolites, in order to maintain and ensure CNS homeostasis [110], [116]–[118]. In fact, while microglia are very important regulators of the CNS inflammatory response, they are also very important controllers of neuronal function [119]. For instance, these, and other glia cells (i.e. astrocytes), provide strength and nourishment to neurons as well as release trophic factors which help maintain cell integrity and function, all in order to ensure neuronal survival [119], [120].

Similar to the cells of the peripheral immune system, microglia have intricate surveillance processes to detect and eliminate pathogens or other threats within their microenvironment [117]. During this surveillance state, these cells have highly ramified processes that constantly monitor their environment and upon activation undergo structural and functional changes that allow for a finely tuned orchestrated response to the potential insult [117]. For instance, injured cells release molecules termed damage associated molecular patterns (DAMPs) (i.e. heat shock proteins, extra nuclear DNA or RNA, chromatin-associated protein high-mobility group box 1; HMGB1) that can activate pattern recognition receptors (PRR) found on microglia, allowing the cells to migrate towards the injury and adopt an active state to engage an appropriate response [121]. Select families of PRRs also detect pathogens via recognition of their unique structure called pathogen associated molecular patterns (PAMPs) also placing these glia cells in an activated state [121]. In regard to compromised neuron(s), once recognized and targeted as being in a damaged or stressed, microglia mobilize and then place the cell in a "clean up" state engaging in processes of debris removal (i.e. phagocytosis) [121]. This "clean up" process may also involve recruiting other microglia (as well as peripheral immune cells) in the vicinity through pro-inflammatory cytokine release, as well as the release of oxidative factors, all while also protecting other neighboring healthy neurons from the damaged tissue [110], [122].

It becomes important to note here that while microglia are largely considered the main immune cells of the CNS, and can as such act in an independent manner, they are highly sensitive to a wide range of peripheral signals [123], [124]. Moreover, it is now accepted that the CNS is actually not solely an immune-privileged environment, and that the resident immune

glia cells can act in conjunction with infiltrating peripheral immune cells to aide in the central neuroinflammatory response [123], [124]. Indeed, it is now well established that brain and peripheral immune interaction is, in fact, fundamental in the maintenance of CNS homeostasis [125]. That is, limited concentrations of peripheral immune cells such as leukocytes normally enter the CNS and often come into close contact with microglia to aide in standard 'house-keeping' tasks [50]. For example, the resident immune cells of the CNS also express MHC cell surface proteins [126]. The expression of MHC under the direction of cytokines, can allow for synaptic interactions between microglia and invading T lymphocytes via receptor recognition and this highly tuned interaction can in turn result in an antigen specific immune response [127].

Nonetheless, it is now very well known that neuroinflammatory processes involving microglia can dramatically affect both health and neurodegenerative diseases [128]. Indeed, under sustained pathological conditions (such as those initiated by genetic predispositions, or recurrent exposure to environmental stressors), and in a diseased state, the continued activation of microglia can contribute to neuronal injury and death through the continuous release of a variety of neurotoxic factors (including cytokines and ROS), and through the recruitment of other immune cells [129], [130]. Importantly, in these states of prolonged neuroinflammation a complex feedback loop can be established that further perpetuates inflammation and that eventually results in neuronal cell death and prolonged neurodegenerative processes [131]. Additionally, cytokines released by microglia can enhance permeability of the BBB through regulation of tight junction proteins (i.e. claudins and occludins), and active transport receptor expression [132], [133] and in turn further recruit mobilization of peripheral immune cells (and

potentially other endogenous toxic agents) across the BBB, which may perpetuate the neuronal damage especially under abnormal, extreme or chronic conditions (i.e. stroke; AD; PD) [134]. Likewise these interactions resulting in compromised BBB integrity can influence the entry of potentially harmful xenobiotics into the brain, which can further engage a neuroinflammatory environment ([135], [136]; for a representative image see figure 2).

Microglia and inflammation: Links to PD

Substantial evidence exists demonstrating that microglia driven chronic inflammation represents an extremely important factor in the progression of PD [137]. This is supported by the fact that, both animal models of PD, and human PD patients, display a number of signs of on-going inflammatory processes at play [3] For instance, augmented microglial reactivity, along with increased levels of pro-inflammatory cytokines (further discussed below), and other inflammatory mediators, are consistently observed in post-mortem brains of PD patients, or in cerebrospinal fluid (CSF) of diagnosed patients [19], [137], [138]. In fact, in comparison to inflammation observed in arthritic joints, the levels of some of these factors appear to be even greater in PD [139]. Additionally, up-regulation of peripheral immune cells including CD4+ and CD8+ T lymphocytes in the nigrostriatal system, has been demonstrated post-mortem, and it has been suggested that peripheral immune factors infiltrate the brain to aide in microglia mediated processes [140]–[145]. In support of this, different treatments used to protect against toxin induced degeneration in PD animal models show that the neuroprotection is coupled with a reduction of microglia, but also T cell infiltration into the midbrain [146], [147]. Finally,

many of the PD-relevant genes including PARK2, *DJ*-1, SNCA, *glucocerebrosidase* (GBA), and LRRK2 are expressed in microglia and other immune cells [148] suggesting that these genes may play a distinct role in the functioning of these cells in response to insult or neuron injury signals [3], [148]. This also raises the notion that alterations in these genes may contribute to an abnormal immune response that could potentially facilitate or exacerbate the microglia-mediated death of SNc dopamine neurons [3].

It should be considered that the augmented central inflammation observed in PD is likely a response that stems from sick/injury signals from affected neurons and as such, engages in mechanisms to promote controlled death or survival [149]. However, it may also be considered that the microglia and central inflammatory processes are actually a primary driving force that contributes to the neurodegeneration observed [149]. This notion is supported by the fact that exposure to a variety of insults that promote a sustained central inflammatory environment have been shown to increase the risk of a clinical PD diagnosis [150]–[152]. Likewise, cell culture and animal PD models validate the fact that microglia mediated inflammatory factors are central in inducing/aiding in the death of dopamine neurons [153], [154]. For instance, rodents treated with MPTP, 6-OHDA, or paraquat have all widely demonstrated augmented levels of activated microglia and pro-inflammatory brain and/or blood cytokine expression, long after the initial insult, and which correlate with cell death observed, suggesting that inflammation is an important mediator in neurodegenerative processes [85]. In fact, as made evident by several *in vitro* experiments, it appears that toxin induced loss of dopamine neurons in culture only occurs in the presence of co-cultured microglia or microglia derived ROS or cytokines [155].

Endotoxin-microglia inflammatory models of PD

As already eluded to, LPS is a highly potent immune response activator used in many models of inflammation and neuroinflammation [156]–[158]. LPS is an endotoxin found in the outer membrane of gram-negative bacteria, that presents a PAMP that is recognized by the PRR known as TLR4, expressed on cells of the innate immune system (i.e. macrophages as well as on microglia and possibly astrocytes) [156], [158]. Endogenously, LPS is mostly derived from the abundant microbiota found in the gastrointestinal tract, where it is thought to translocate through the mucous permeable membrane and the gut lumen it surrounds under conditions of enhanced permeability [159]. Whatever the case, upon recognition of the endotoxin by TLR4, a downstream intracellular signaling cascade is induced leading to immune cell activation [158]. For instance, endotoxin-receptor interaction on microglia allows these cells to migrate towards the pathogen, and sequentially undergo a phenotypic shift in which these active microglia can become phagocytic and release a variety of noxious factors including pro-inflammatory cytokines, and ROS [158]. As an example, intracellularly TLR4 stimulation can initiate activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) dependent pathways, which result in release of pro-inflammatory cytokines, and can in turn further activate and recruit other immune cells [156], [158].

Intriguingly, over-activation of TLR4 has been associated with neurodegenerative diseases including PD [160]. Indeed, evidence has demonstrated modulated expression of this receptor in post-mortem brains of patients and in toxin-induced animal models [161]–[163].

Moreover, direct exposure to relatively high levels of LPS in the SNc results in not only augmented levels of activated microglia and pro-inflammatory cytokines, but also a reduction of midbrain dopamine neurons supporting the idea that inflammation can act as a primary driving force of SNc neuron degeneration [164]. In fact, this is also supported by a study demonstrating that a single LPS injection into the cavity can promote microglia activation that persists for 10 months and results in delayed and progressive loss of SNc dopamine neurons, suggesting that a single LPS dose can promote sustained self-propelling neuroinflammation that has detrimental consequences [154]. Interestingly, central LPS administration to areas outside of the nigrostriatal system (i.e. hippocampus, thalamus and cortex) has been shown to produced little cell loss [165], [166]. Importantly, there is a relatively high concentration of microglia observed in the SNc (in opposition to other areas) [166], [167]. Given that SNc dopamine neurons are very metabolically active, when exposed to immune stimulating agents like LPS, the relatively high numbers of microglia may enhance the already vulnerable neurons in the area [166], [167].

Additional PD studies attempting to more realistically model the multi-hit nature of the disease have also shown the importance of a central inflammatory environment [31], [46], [168], [169]. These studies have focused on combining the effects of dopamine-related toxins (i.e. 6-OHDA, paraquat, rotenone) in combination with immunogenic challenge, such as LPS [46], [168], [170], [171]. For example, similar to a psychological stress challenge, if you prime the central inflammatory environment with LPS, you can sensitize the microglial response to subsequent toxins, and in fact cause augmented cell death [46], [171]. Indeed, our own lab has

shown augmentation of the neurotoxic effects of paraquat 2 days following intra-nigral administration to a relatively low dose of LPS [46]. In fact, the enhanced SNc degeneration observed occurred when paraquat administration began at the time point when the microglial response was most active following administration of the TLR4 agonist (i.e. 2 days), suggesting that LPS administration primed the microglial response to be more sensitive, and as such contributed to the degenerative cascade induced by later paraquat exposure [19], [46]. Intriguingly, if paraquat administration was given outside of this window of highly reactive microglia, the augmented cell death was not observed, and when the toxin was given 7 days following central administration of LPS when microglia was least active, SNc dopamine neurons were actually protected from the toxin induced cell loss [46]. This study illustrates that the state of the brains microenvironment is important, as is the time between stressor challenges. Of course this holds true with other challenges as well such as psychological stress exposure or ischemic stroke. For example, if an ischemic event is induced 30 minutes following LPS, augmented damage is evident [172], however if the ischemic event is given 24 hours following LPS treatment, reduction in damage is observed [173].

We also demonstrated that mice who received intra-nigral injections of the double-stranded RNA viral analog, polyinosinic: polycytidylic acid (poly(I:C) (a TLR3 agonist) prior to paraquat exposure, had enhanced SNc dopamine cell loss and microglia activation in comparison to receiving the toxin on its own [168]. Thus, both bacterial and viral-like toxins appear to be capable of sensitizing microglial reactivity and in turn, increased dopaminergic neuronal death. Indeed, this is consistent with the "multiple hit" model of PD, which as

previously stated suggests that initial events or "hits" can enhance the vulnerability of neurons by priming biological systems to be more readily reactive to toxic insult [23], [34].

Cytokines and inflammation: Links to PD

One important player that regulates peripheral and central immune processes, as well as the communication between the two systems, includes cytokines [79]. Measured in concentrations of picomolars and nanomolars, these small glycoproteins transmit messages amongst immune cells regarding the presence of pathogens or injury [79]. Moreover, cytokines have been shown to regulate not only inflammation, but also trigger a cascade of intracellular signaling events that regulate a vast array of cellular functions including cell to cell adhesion, cytokine secretion, differentiation, proliferation, survival, and death [79]. For instance, in response to infection or injury, chemokines (a class of cytokines), attract immune cells to the injured site and further direct these cells to release a variety of inflammatory factors including pro-inflammatory cytokines and oxidative molecules [85], [174]. In addition to chemokines, other recognized cytokines include the interleukins (ILs), interferons (IFNs), and tumor necrosis factors (TNFs) [175]. In general, these immunotransmitters are broadly divided into one of two categories: pro-inflammatory and anti-inflammatory, however classification may be overly reductive as in many cases cytokines act in a pleiotropic and synergistic fashion [175]. Whatever the case, cytokines including IL-1β, IL-6, TNF-α, and IFN-α/β/γ are largely classified as pro-inflammatory, while IL-2, IL-4 and IL-10 fall into the anti-inflammatory realm.

Within the CNS microglia, oligodendrocytes, astrocytes and neurons themselves are all known to synthesize and respond to cytokine signaling [176]–[178]. In fact, in the brain, cytokines serve a number of critical functions acting as neuromodulators regulating neurodevelopment and synaptic transmission, in addition to neuroinflammation [179]. In regard to neuroinflammation, cytokines are capable of not only activating and attracting microglia and peripheral immune cells to the site of injury to promote and sustain inflammation and repair [180], [181] but are also able to act as autocrine signals that sustain activation of the cell they were released from [182]. Likewise, as previously mentioned, these protein messengers can influence BBB permeability through interacting with brain vasculature to modify tight junction proteins and receptor transport expression [180], [181]. Additionally, cytokine signaling can coordinate the infiltration of peripheral immune factors (i.e. adaptive immune cells including T cells and B cells) to aide in central inflammatory response [134].

Upon recognition by their receptor, cytokines largely act via initiation of either the (1) signal transducer and activator of transcription (STAT) pathway, (2) the c-Jun N terminal kinase (JNK) pathway, or (3) the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway [19], [183]–[185]. Cytokine activation (i.e. by IL-6, IL-10, IFN-γ) of the JNK and STAT pathway typically result in production of highly imperative proteins involved in not only inflammatory processes but also additional processes important for intracellular and neuronal functioning [186]. In regard to activation of the NF-κB pathway, a carefully orchestrated response results upon ligand recognition which engages the breakdown of the inhibitory factor, IκB, which normally prevents the transcriptional actions of NF-κB [184].

Once degradation of IκB occurs, NF-κB is able to translocate from the cytoplasm to the nucleus where it influences the expression of inflammatory genes [184]. For example, our own lab (and others) have demonstrated that production of the inflammatory enzyme, cyclooxygenase-2 (COX-2), occurs following NF-κB activation [187]. Interestingly, global ablation of COX-2 alters pro-inflammatory cytokine expression upon exposure to endotoxin or stressor challenges [187].

Research involving cytokines over the last three decades has generally portrayed pro-inflammatory cytokines as having deleterious influence within the brain. Although it should be noted that this statement is somewhat complicated at times by the fact that emerging research now paints cytokines as playing more than one role in neurodegeneration and neuroprotection [188]. For instance, the often classified pro-inflammatory cytokine IL-6 is released as a response to neuron injury in order to promote survival [189]. However, while this cytokine indeed has trophic properties [189], it is also negatively associated with a number of neurodegenerative diseases [190]–[192]. In fact, high levels of IL-6 and other pro-inflammatory cytokines (i.e. IL-1β, TNF-α, and IFN-γ) are often linked to a variety of brain pathologies including Amyotrophic lateral sclerosis (ALS), AD, and PD [83], [193], [194]. Conversely, the use of anti-inflammatory treatment agents including non-steroidal anti-inflammatory drugs (NSAIDS), which indirectly target inflammatory cytokines, have been shown to be neuroprotective in animal models, and it has even been suggested that use of these drugs may be protective in humans [195], [196].

In regard to PD, genetic studies have demonstrated that polymorphisms in IL-1β and TNF-α, increase risk for disease development [197]–[200]. As an example, carriers for either or both polymorphisms in the IL-1β and TNF-α genes encoding for pro-inflammatory cytokines have an approximately 3-fold risk of developing the disease [200], [201]. Animal models in which select pro-inflammatory factors (i.e. IL-1, TNF-α or IFN-γ) have been ablated, have also widely demonstrated protection against toxin induced nigrostriatal damage [165], [202]–[204]. For example, global ablation of IL-1 was shown to protect against LPS induced dopamine cell loss and motor-function decline [165]. Likewise, genetic knock-out of TNF-α or IFN-γ was shown to protect against the inflammatory and neurodegenerative effects of acute MPTP administration [205]. In fact, these authors report that both TNF-α and IFN-γ are critically important for the sustained microglial recruitment that occurs prior to cell death [205]. These findings are similar to our own group who found that IFN-γ ablation protected against microglia activation, neurochemical changes and dopamine neuron loss in the SNc induced by paraquat exposure [204], [206].

Pro-inflammatory cytokines have been implicated in a wide number of pro-oxidant and excitotoxic pathways that eventually result in neuronal cell death. Indeed, a number of these inflammatory signal proteins can induce expression of oxidative enzymes (i.e. inducible nitric oxide synthase (iNOS); NADPH) [207] that eventually result in free radical production (i.e. superoxide and peroxynitrate) that when sustained culminates in oxidative damage to lipids, protein, and DNA, propagating the activation of cell death cascades [208]. For instance, cultured human neuroblastoma cells treated with IFN-α has been shown to induce apoptosis (as

measured by the BAX2: Bax:Bcl-2 mRNA ratio) that is in part due to augmented levels of ROS induced by treatment with the cytokine [209]. In fact treatment with N-acetyl-cysteine, a highly potent antioxidant and mitochondrial modulator, was able to counteract induction of the cytokine induced apoptotic activation [209]. Alternatively, while such cytokines can cause cell death through the release of oxidative factors, they can also directly activate intracellular cell death pathways [210]. Cytokines like TNF-α for example can directly silence intracellular survival signals and concomitantly induce apoptotic caspase signaling through interaction with its endogenous neuronal p55 receptor [210]. While cytokines like IL-1β can induce excitotoxic neuronal cell death through elevated glutamate levels [210]. Whatever the case, in an uncontrolled environment, cytokines are important mediators of degeneration [210].

Inflammatory driven oxidative stress and PD

Oxidative stress is generally defined as the production or accumulation of ROS and concurrent failure of antioxidant metabolites or enzymes to effectively deal with these free radicals [37], [211]. As such, this imbalance can result in an environment that favors free radical production that can lead to a wide range of deleterious intracellular effects, that can eventually result in cell death [211], [212]. Importantly, concomitant with elevated immune factors, post-mortem brains of PD patient's consistently exhibit elevated levels of oxidized lipids, damaged proteins, and DNA, as well as alterations in antioxidant levels (i.e. reductions in glutathione (GSH), and augmented superoxide dismutase; SOD) suggesting a central role for oxidative stress in the pathology of the disease [211], [212]. It is highly likely that the drivers of ROS

production include neuroinflammation (i.e. microglia release of these factors) as well as intrinsic intracellular processes, including mitochondrial dysfunction, and dopamine metabolism [37], [211].

As already mentioned, the activation of microglia not only causes the release of inflammatory cytokines, but also ROS as a natural defense mechanism against cellular threats [207]. For instance, inflammatory signals can induce the expression of oxidative enzymes (i.e. iNOS; NADPH oxidase) on microglia [207]. In fact, our own group found that paraquat induced NADPH oxidase activation on microglia, causing the release of superoxide, which is known to react with other enzymes to initiate damage to DNA and other proteins [168], [213]. Moreover, ablation of different subunits required for NADPH activation (i.e. gp91 or $p67^{PHOX}$) protected against 6-OHDA, MPTP and paraquat induced cell loss [214]–[216] and these subunits have been shown to be elevated in PD patients [214].

Intriguingly, SNc dopamine neurons are particularly sensitive to effects of ROS not only because of their high rate of oxygen utilization and density of microglia, but also due to the fact that this brain region has especially low levels of antioxidant enzymes (i.e. SOD, GSH, CAT) [217]. In addition, the intracellular processes themselves that are involved in the synthesis, storage, and breakdown of dopamine in these cells can result in the generation of ROS [59], [218]. Indeed, it is well known that storage of dopamine into synaptic vesicles occurs by the vesicular monoamine transporter 2 (VMAT2) in order to prevent excessive levels in the cytosol [219]. When inside the vesicle, dopamine is relatively stable, however, when the monoamine

is present in the cytosol (i.e. outside of these vesicles), it is readily metabolized by monoamine oxidase (MAO) [219]. Furthermore, dopamine present in the cytosol can also undergo auto-oxidation in the oxygen rich environment, resulting in the formation of superoxide, as well as cytotoxic quinones [59]. Likewise, evidence demonstrates that the high concentrations of iron observed in the SNc, can react with dopamine and neuromelanin to form a "toxic couple", and may represent an upstream mechanism that results in cell death observed in the disease [220]. Whatever the source of ROS, if antioxidants (i.e. SOD; GSH; CAT) present in the mitochondrial or cytosol are unable to effectively deal with extensive damage, a self-propagating chain of ROS can damage proteins, lipid membranes and nuclear DNA leading to cell death [59].

In sum, under normal conditions the central inflammatory environment involving cytokine signaling and ROS production is an evolutionary conserved and extremely imperative response that serves a number of critical functions including the promotion of CNS tissue repair, the effective elimination of pathogens, as well as removal of cellular debris [221]. However, evidence now clearly demonstrates that these processes can have extremely detrimental consequences when protracted or not properly regulated [221]. That is, when not under appropriate control (i.e. due to an inability to effectively deal with initial or sustained triggers), the continued release of oxidative-inflammatory factors can result in sustained microglia mediated inflammatory environment that leads to neuronal damage and dysfunction observed in PD [130], [149], [221], [222].

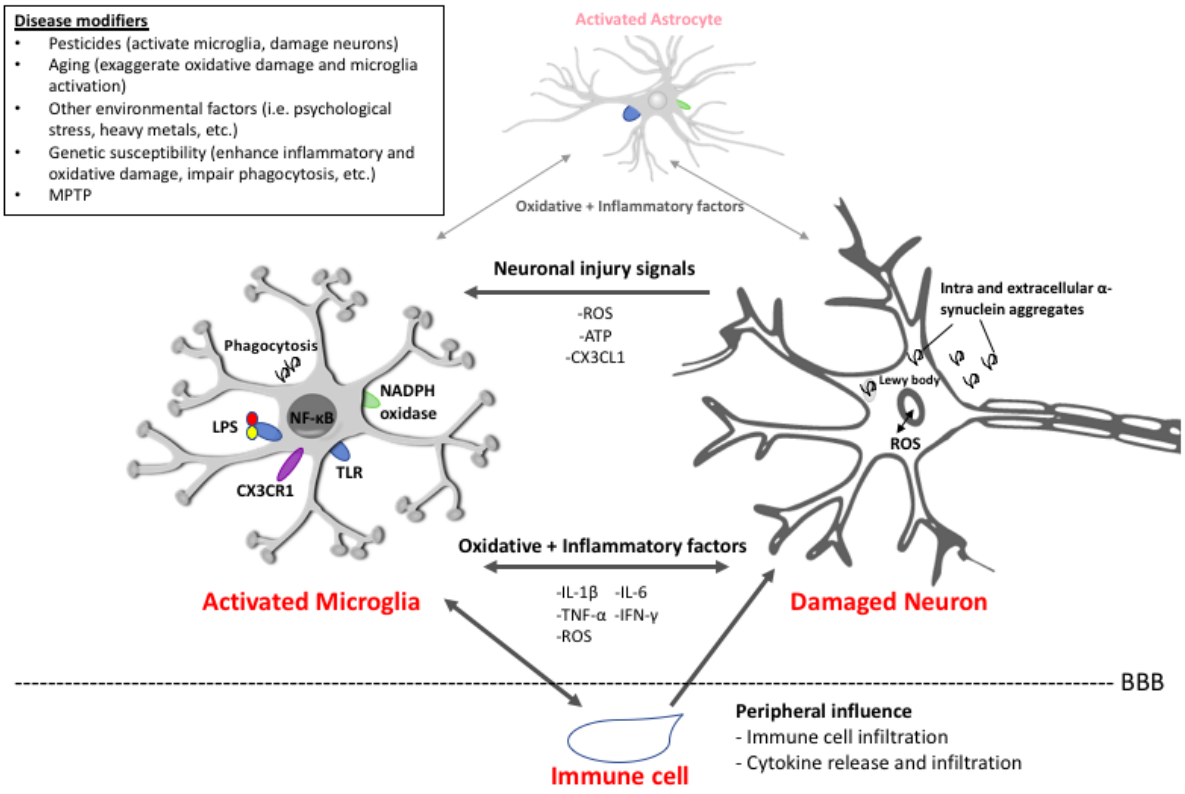

Figure 2: Microglia mediated neuroinflammation in Parkinson's disease.

Parkinson's disease (PD) is characterized by loss of dopamine neurons in the substantia nigra pars compacta (SNc) as well as the presence of intracellular inclusions containing α-synuclein protein aggregates, called Lewy bodies. In addition to their presence in Lewy bodies, α-synuclein aggregates, as well as the endotoxin lipopolysaccharide (LPS) can activate Toll-like receptors (TLR) present on microglia. This activation leads to NF-κB mediated transcription and the sequential release of pro-inflammatory mediators including pro-inflammatory cytokines as well as the release of reactive oxygen species (ROS) through NADPH dependent mechanisms. Damaged neurons also release injury signals (i.e. fractalkine (CX3CL1) or ATP). Injury signals like ATP can cause microglia activation also triggering the release of oxidative-inflammatory factors. Importantly, the release of these factors from microglia, potentially astrocytes, and neurons themselves can further damage dopamine neurons in the SNc. Furthermore this inflammatory response is likely mediated by cytokine directed CNS infiltration of peripheral immune cells. Additionally the exaggerated immune response may be further enhanced/induced by the interaction between other disease modifiers such as genetic

abnormalities, environmental factors (i.e. pesticide exposure or stress exposure, and aging itself). Whatever the case, sustained microglia activation and release of oxidative-inflammatory factors results in the induction of a positive feedback loop that further enhances an inflammatory environment and neurodegeneration (Image modified from Glass et al., 2010 [223])

Leucine-rich Repeat Kinase 2 (LRRK2) and PD

While it is recognized that the vast majority of PD cases are sporadic in nature (representing 90% of cases), familial studies have identified over 15 genes as potential causal factors [224], [225]. Of these genes, coding variations present in the LRRK2 gene, represent the most frequent mutations associated with familial forms of the disease [226]. In fact, a number of LRRK2 mutations and polymorphisms are also associated with sporadic late-onset forms of the disease, potentially present in up to 10% of idiopathic cases [227]–[230]. As previously stated, PD appears to be a highly heterogeneous disorder with autosomal dominant/recessive familial forms (e.g., LRRK2, DJ-1, Parkin) at one end of the spectrum, and purely environmental "toxic exposure" cases at the other [19]. Consequently, the bulk of "idiopathic" cases fall in the middle and likely involve a mix of genetic and environmental influence [19]. In regard to LRRK2, research demonstrates that it is highly likely that associated coding changes can represent either a dominant genetic factor that causes early onset (i.e. before the age of 60) forms of disease, or alternatively, represent a vulnerability factor in which its interaction with some other insult(s) results in forms of the disease that presents itself later on in life [231]. Indeed, heterozygotic carriers of LRRK2 mutations don't always show the behavioral phenotype or go on to develop PD [232]. Moreover, among those diagnosed PD patients with an inherited LRRK2 mutation

their clinical and neuropathological symptoms have a high degree of variability (similar to idiopathic PD) suggesting (1) low and variable penetrance of the gene, and (2) that these mutations may be susceptibility factors that interact with environmental variables to induce PD pathology [29], [85]. As an example, despite the high prevalence of the G2019S mutation in the LRRK2 gene, there is incomplete penetrance, and it appears that it is age-dependent suggesting that age related deficits, coupled with exposure to environmental insults, may contribute to the forms of the disease involving this genetic abnormality [233]. This provides support from our own lab (and others) who have commonly promoted the notion that environmental events, such as exposure to environmental stressors, may be important modifiers or triggers of LRRK2-related PD pathology [234].

Highly conserved across species, the LRRK2 protein (formally known as dardarin) is a relatively large (286-kDa) complex protein that belongs to the RoCo super family of proteins [235], [236]. Its structure consists of protein-protein interaction domains including armadillo repeats (ARM), ankyrin repeats (ANK), leucine-rich repeats (LRR), present at the N-terminus, and a WD40 domain in the C-terminus [236]. Also present in its architecture includes two enzyme domains: a ROCO GTPase supradomain which incorporates a Ras of complex proteins (ROC) and C-terminal of ROC (COR) domains; as well as a serine/threonine kinase domain [236] (See Figure 3). To date, over 100 different mutations in LRRK2 have been identified to impact PD susceptibility of which six have demonstrated robust evidence for being involved in PD pathogenesis [228]. While not yet clear of the precise role of these mutations, given their location primarily within the enzymatic domains, it has strongly been suggested that

dysfunction in kinase activity which controls a number of intracellular signalling pathways, is what underlies LRRK2-PD related pathology [236].

Of the mutations associated in PD, the most common is the G2019S mutation present on the kinase domain, which is seen in approximately 4-8% of hereditary and 1-3% of sporadic cases [29], [237]–[239]. In fact, in select populations such as North African Arab Berber and Ashkenazi Jewish populaces, familial transmitted LRRK2 mutations are as high as 20% in clinically diagnosed patients [240], [241]. Whatever the case, it appears that the majority of individuals who possesses pathogenic coding variations in LRRK2 display symptoms of PD which are almost identical to sporadic cases (i.e. proteinaceous inclusions, late-onset, cardinal motor deficits) and together findings suggest that LRRK2 may be a critical modifier of some of the underlying neurobiology in sporadic and familial related forms of the disease [242].

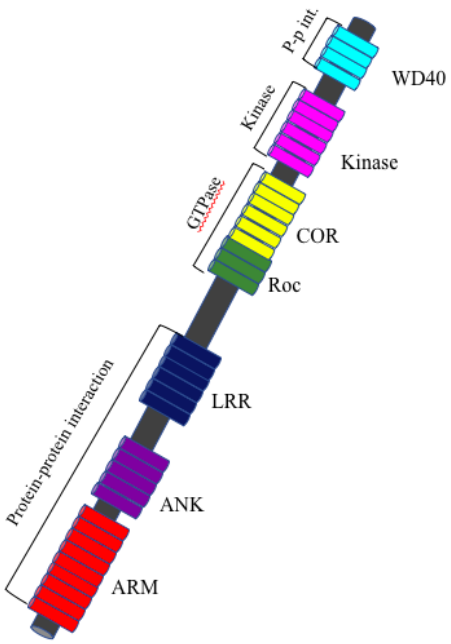

Figure 3: Human LRRK2 protein sequence.

LRRK2 domains are as follows: Shown in red is the armadillo-like repeat, ARM; shown in purple is ankyrin repeat, ANK; shown in blue is leucine-rich repeat, LRR. The later represent protein-protein interaction domains. Shown in green is the Roc (ras of complex) GTPase domain, GTPase; shown in yellow is that C-terminal of Roc, COR; shown in pink is the kinase domain; shown in light blue is the WD40 domain, also a protein-protein interaction domain.

LRRK2 immune involvement

Since the discovery of the association between LRRK2 and PD in 2004 [243], substantial research has gone into elucidating the role of the protein and associated mutations under normal and stress conditions [229]. Accordingly, given its size and multi-domain structure, it has been shown to be involved in a plethora of biological functions (for a review see [229], [236], [244], [245]). For instance, intracellularly, LRKK2 is thought to control a number of signalling

pathways as it interacts with a wide array of structures and additional proteins including synaptic/transport vesicles [246], endosomes [247], lysosomes [248] as well as the endoplasmic reticulum [249], mitochondria [250], and the golgi apparatus [251]. As such, mechanistically, LRRK2 is believed to play a prominent role in a plethora of intracellular functions including regulating cytoskeletal organization [252], protein regulation [236], vesicular trafficking [253], and apoptosis [254]. Nevertheless, to date, the physiological function(s) of "normal" LRRK2 and associated mutations still remain to be fully determined, and there is a need for consistent reproducibility of recent findings [3].

Intriguingly, over the last decade substantial evidence has come out to support LRRK2 involvement in inflammatory processes [24]. Genome-wide association studies (GWAS) have not only linked LRRK2 to PD susceptibility, but also other conditions with a prominent immune-inflammatory component including the inflammatory bowel disease, Chrohn's, as well as the infectious disease Leprosy [255]–[257]. Following the implication of LRRK2 involvement in inflammatory disease, it was proposed that LRRK2 may also be involved in modifying the inflammatory pathway relevant to neurodegeneration [257]. Indeed, in addition to the presence of LRRK2 in neurons [258], there is also particularly high LRRK2 expression in immune cells such as dendritic cells [259], B lymphocytes [259], [260], monocytes [261], [262], astrocytes [258], and microglia [258]. In fact, expression of LRRK2 is three to four times higher in microglia than in neuronal cells, suggesting a major role for the protein in the inflammatory response processes of these CNS resident macrophages, which may be critical for neurodegeneration observed in PD [263], [264].

Accumulating evidence strongly suggests that LRRK2 plays a modulatory role in microglia activation, and aides in driving these cells to adopt a more reactive phenotype [257], [265]. Indeed, in response to a variety of immune stimulating agents (e.g. LPS or IFN-γ) LRRK2 has been shown to play a role in cytokine release, migration, and phagocytic properties of these cells (for a review see [3]). For instance, a marked increase of LRRK2 protein is observed in primary microglia cell cultures following stimulation with the inflammatory cytokine IFN-γ or LPS [266], as well as in microglia in the SNc and striatum of mice exposed to the endotoxin [257]. In regard to cytokine release, LRRK2 has been shown to regulate intrinsic response processes in microglia including NF-κB-dependent transcription, effectively modulating pro-inflammatory cytokine activity [164], [259], [264], [267]. Indeed, silencing LRRK2 in stimulated microglia attenuates NF-κB transcription as well as expression of iNOS and COX-2, and the release of the pro-inflammatory cytokines IL-6, TNF-α, and IL-1β [257], [264], [267], [268] Support for the notion that LRRK2 may be involved in other inflammatory related intracellular pathways involved in microglia reactivity also comes from LRRK2 regulation of other transcription factors associated with the inflammatory response [269]. For instance, LRRK2 has also been shown to partially regulate the nuclear factor of activated T cells (NFAT) transcription factor, which typically targets genes encoding pro-inflammatory cytokines including IFN-γ, and IL-6 [265], [269].

In addition to regulating molecular pathways important for cytokine release, recent evidence also suggests that LRRK2 may be involved in the rapid cytoskeletal reorganization required for microglia to appropriately respond to injury/threat [265], [270], [271]. Indeed this

notion is supported by the fact that, in response to various inflammatory agents (i.e. LPS, HIV-1 tat protein), microglia fail to adapt an active appearance (characterized by an amoeboid like shape in that lacks extended branch like processes) in LRRK2 null rodents, or when inhibiting LRRK2 kinase activity [257], [272], [273]. Interestingly, LRRK2 deficient rats have demonstrated protection against LPS induced cytotoxic microglia and subsequent SNc dopamine cell loss [257]. Conversely, the nigrostriatal pathway is actually hypersensitive to the endotoxin challenge in G2019S BAC transgenic (Tg) rats, effects which are perhaps due to dysregulated LRRK2 control of cytoskeletal reorganization of microglia to appropriately respond (coupled with dysregulated control of the release of cytotoxic factors) [164], [257], [274]. Likewise, cell culture models have demonstrated that, in response to different chemoattractants (i.e. ADP, thioglycolate; LPS), microglia display severely impaired movement/motility (which requires actin cytoskeleton reorganization) when LRRK2 was pharmacologically ablated [257], [275]. In fact, LRRK2 has been shown to interact with a number of actin-regulatory proteins, suggesting that the protein may regulate actin dynamics required for motility, migration, activation, and even phagocytosis in response to different challenges [274], [276]. However, despite these observations, the underlying mechanisms are still unknown [264]. Whatever the case, it appears that LRRK2 is a critical for the inflammatory response [3]. However, whether or not LRRK2 plays a role in paraquat induced toxicity, or microglia mediated dopaminergic cell death induced by paraquat or LPS + paraquat, has yet to be determined.

This thesis is encompassed primarily by two overarching themes including 1) Evaluating the interactive or independent impact of different types of stressors (including psychological, immune, and aging) on the behavioral and biological PD related features induced by the chemical toxicant paraquat and 2) Providing further understanding of the role of LRRK2 as a mediator of the SNc neuroinflammatory processes evoked by paraquat or LPS. Accordingly, this thesis comprises three major experiments. In brief, Chapter 1 focuses upon whether a chronic unpredictable stressor regimen that is known to provoke depressive/anxious symptoms, renders mice more vulnerable to the impact of paraquat exposure. Chapter 2 (studies 2 and 3) utilize an immune stressor, LPS; again asking the question as to whether this insult enhances the impact of later paraquat exposure. Chapter 3 (Study 4) uses mice of more advanced age (~8 months, compared to 3 in the other studies), to ascertain whether this factor might also impact the toxic effects of paraquat exposure (not only with regards to the brain, but also at peripheral sites). Finally, a key element of this thesis is whether inflammatory mechanisms underlie the deleterious effects of the aforementioned insults. As already discussed, we believe that LRRK2 is a crucial mediator of PD-like effects in these animal models. Hence, LRRK2 knockout mice (and their wild type littermates) were used in Studies 2-4.

Chapter 1. Chronic unpredictable stress differentially influenced impact of paraquat upon behavioral and neuronal outcomes.

Preface

Animal models of PD have traditionally focused the primary motor characteristics of the disease. Of course, given that PD is a multi-system disorder, it is important that these models also involve the non-motor behavioural characteristics that are commonly comorbid in the disease [277]. As stated in the general introduction, some of these co-morbid features occur before the observance of the primary motor features and may represent a possible risk factor for the disease [278]. Indeed, it is thought that the motor behavioral features of the disease only become evident after 50%-80% of dopamine neurons in the SNc have already been lost, so identifying early behavioral and biological characteristics of the disease may help to halt or slow its progression [21], [22].

Whatever the case, non-motor behaviors, and in particular, neuropsychiatric disturbances including the two most common symptoms, anxiety and depression, pose a major threat to quality of life among PD patients [278]–[281]. In fact, while of course not unique to PD (similar to other neuropsychiatric disturbances), depression is thought to impact quality of life exceeding that of the primary motor symptoms [282]. Interestingly this neuropsychiatric disorder is said to occur more in PD than in any other disorder including multiple sclerosis (MS) and Alzheimer's disease (AD) [280]. While estimates of depression in PD generally range from 30-50%, it is likely that many cases remain clinically undiagnosed due to characteristics being mistaken for those of motor deficits [278], [281]. Indeed, when taking this into consideration, estimates of depression in PD are as high as 90% [281]. Accordingly, one of the

main goals of our research study outlined below was to seek to demonstrate whether or not the environmental toxicant, paraquat, induces select non-motor behavioral alterations, as typically associated with PD, including neuropsychiatric disturbances (i.e. depressive and anxiety-like characteristics) as well as cognitive alterations. In addition, we also sought to further characterize the biological effects of this toxin outside of the nigrostriatal system.

Intriguingly, previous research from our lab has demonstrated that paraquat has effects similar to those typically observed with chronic psychological stressor exposure [283], [284]. In particular, the herbicide induced neurotransmitter alterations in stress sensitive brain regions and increased plasma corticosterone levels [283]–[285]. Moreover, there is some evidence to suggest that the toxin may induce behavioral deficits similar to those induced by exposure to chronic stress [283]. As such, another goal of the study described in Chapter 1 of interest to us was to further assess whether paraquat itself acts as a systemic stressor, having stressor-like consequences both behaviorally and at the biological level.

Given the growing support surrounding the multi-hit hypothesis for PD, one of the themes of the current thesis is the characterization of how different stressors (i.e. psychological, immune, aging) may interact with chemical toxicants to influence behavioural and biological features of PD in mice. As such, it is well known that chronic psychological stress can engender a variety of biological alterations (including microglia activation, reduction in trophic support, etc.) that may render the brain susceptible to the effects of different toxins such as pesticide exposure [77]. While we have previously shown [283] that paraquat exposure is largely

unaffected by concomitant exposure to chronic mild stress (given only 2X/week given on the same day as paraquat) in the case of depressive-like behaviors (i.e. anhedonia), the present study used a more robust stressor regimen and began the stress exposure three weeks prior to the commencement of the paraquat injections. As well, the present study sought to evaluate a range of behaviors greater than what we previously have assessed, including thorough examination of whether or not stress exposure influences paraquat induced motor behavioral deficits.

As such, the current study sought to (1) further establish whether paraquat exposure influences non-motor behaviours, (2) determine if exposure to the toxin results in stressor-like consequences as suggested by previous studies and (3) to ascertain whether the impact of the pesticide might be enhanced in the context of chronic unpredictable stress exposure on motor and non-motor behavioural measures, and accompanying dopaminergic neuronal degeneration. We hypothesized that mice exposed to chronic unpredictable stress prior to and during paraquat treatment will have enhanced motor behavioural disturbances that are accompanied by augmented dopamine neuron loss in the SNc and greater signs of microglial activation in the area.

Highlights

- Paraquat induced behavioral (i.e. cognitive, anxiety, and depressive) and neurochemical alterations (HPA activity and BDNF expression) similar to those induced by a chronic unpredictable stress regimen.
- Chronic unpredictable stress exposure did not influence the degeneration of midbrain dopamine neurons or accompanying microglia activation in the area induced by the toxin; however, it did influence motor coordination.
- Chronic unpredictable stress exposure did not influence paraquat provoked elevations in corticosterone, however did influence hippocampal glucocorticoid receptor expression in response to the toxin.

Abstract

The impact of psychological stressors on the progression of motor and non-motor disturbances observed in Parkinson's disease (PD) has previously received little attention. Given that PD likely results from many different environmental "hits", we were interested in whether a chronic unpredictable stressor regimen would augment the impact of the toxicant, paraquat, which has previously been linked to PD. Our findings support the contention that paraquat itself acted as a systemic stressor, with the pesticide increasing plasma corticosterone, as well as altering glucocorticoid receptor (GR) expression in the hippocampus. Furthermore, stressed mice that also received paraquat exposure displayed augmented motor coordination impairment but not home cage activity or enhanced signs of degeneration. Yet, the individual stressor and paraquat treatments caused a range of non-motor (e.g. sucrose preference, open field, Y and plus mazes) deficits, but were limited signs of an interaction (additive or synergistic) between these insults. Taken together, these results confirm that paraquat has many effects comparable to that of a more traditional stressor, but does not readily interact with an ongoing stressor to modulate pathology. Ultimately, these data support the notion that paraquat likely affects stress circuitry through distant mechanisms different from psychological insults.

Introduction

Primarily characterized by the loss of dopamine neurons in the substantia nigra pars compacta (SNc), Parkinson's disease (PD) is the second most common neurodegenerative disease [1], [2]. Current evidence suggests that PD pathogenesis involves a complex interaction between a number of risk factors including genetic susceptibility, age, and environmental factors (i.e. sustained exposure to psychological stress and/or exposure to chemical stressors like pesticides) that give rise to sporadic forms of the disease [31], [51]. The link between PD and cumulative lifetime pesticide exposure has been supported for some time [26], [286], with studies showing chronically exposed plantation and farm workers having an increase likelihood of developing the disease [287], [288]. In particular, evidence suggests that the pro-oxidant herbicide, paraquat, can provoke neurodegeneration and other PD associated biological alterations (i.e. oxidative stress, pro-inflammatory milieu, Lewy-body like α–synuclein dense aggregates) within the nigrostriatal system (the principal region affected in PD patients) [49], [65], [277], [289]–[291]. Recent evidence also suggests that the herbicide may be particularly germane for some of the non-motor features of the disease including select neuropsychiatric disturbances (i.e. anxiety and depression), as well as cognitive impairment [284], [292]–[294]. Indeed, paraquat is widely distributed throughout the brain including the olfactory bulbs, prefrontal cortex, and hippocampus, where it can impart a variety of neurochemical changes that may, in part, explain some of these behavioral deficits [51], [55], [295]. However further

characterization of paraquats effects on non-motor behaviors and biological alterations outside of the nigrostriatal system is required.

It is highly likely that paraquat acts in concert with a number of environmental factors in order to give rise to or augment the presentation PD symptoms [26]. In line with this notion, it is now known that even psychologically relevant stressors may impact motor and non-motor manifestations seen in PD patients [296]. For instance, major life events have been shown to impact the development of depression in patients [104], and patients report worsened tremor when in an anxious state [296]. In fact, it has been shown that prolonged psychological stress exposure can impart dysregulation in a number of biological responses known to be involved in PD, including oxidative stress, and neuroinflammation, which can have critical implications on nigrostriatal degeneration [297]. As an example, prolonged stress exposure has been shown to enhance inflammatory and oxidative-stress responses, and neuronal loss [76], [100], [102], [103], [298]–[301]. Indeed chronic unpredictable stressor exposure exaggerated the nigrostriatal damage and motor behavioral impairments induced by the PD relevant toxicants, MPTP or 6-OHDA, [76], [100], [102], [103]. However, whether or not chronic stress exposure influences motor symptom presentation in response to paraquat has yet to be determined.

Intriguingly, previous research from our lab has demonstrated that paraquat acts in a manner similar to chronic psychological stress in mice imparting hypothalamus-pituitary adrenal (HPA) axis activation, as well as inducing neurochemical alterations in a variety of stress sensitive brain regions (i.e. hypothalamus, hippocampus, central amygdala), supporting

our contention that paraquat itself acts as a systemic stressor [283], [285]. As such, part of the study outlined below was to further validate this notion by looking at the toxin's effects on a variety of non-motor behaviors (i.e. cognitive and neuropsychiatric) and associated neurochemical alterations known to be altered by exposure to chronic unpredictable stress by directly comparing paraquat exposure with a psychologically relevant chronic unpredictable stress regimen. Furthermore, we were interested in evaluating whether or not chronic stressor exposure would alter any of the behavioral (both motor and non-motor) and chemical changes induced by the toxin. We hypothesized that mice exposed to chronic unpredictable stress prior to and during paraquat treatment will have enhanced motor and non-motor behavioural disturbances that are accompanied by augmented dopamine neuron loss in the SNc and greater signs of inflammation (microglial activation) in the area.

Methods

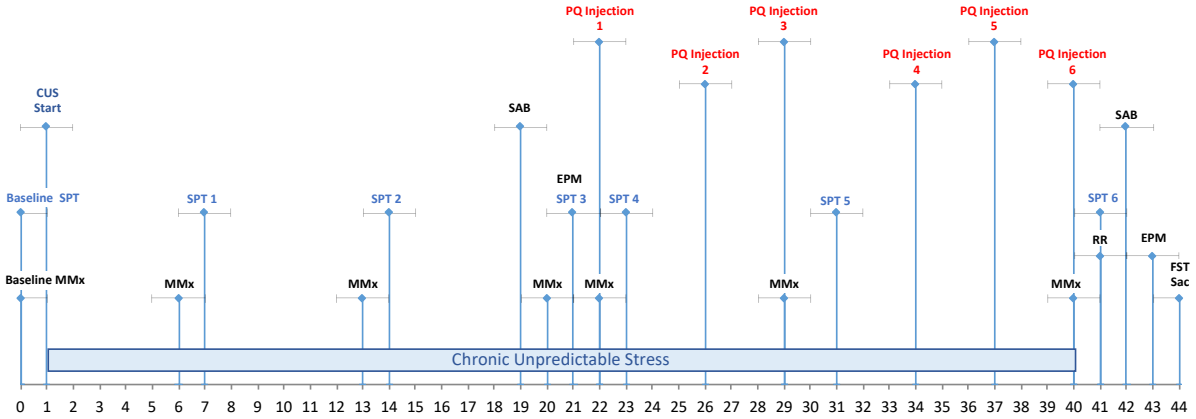

Figure 1: Schematic timeline of study

Schematic timeline (CUS = chronic unpredictable stress; EPM = elevated plus maze; FST = forced swim test; MMx = Micromax; PQ = paraquat; RR = rotarod; SAB = spontaneous alternation behavior Y maze; SPT = sucrose preference test).

Animals and general experimental design

A schematic timeline for the experiment can be observed in Figure 1. Fifty-six male C57BL6/J mice were obtained at 3 months of age from Charles River Laboratories Montreal, QC, CAN) and were acclimated to our vivarium for 2 weeks prior to the start of the experiment. All animals were singly housed in standard polypropylene cages (27 × 21 × 14 cm) and maintained on a 12-h light/dark cycle with lights on at 08:00 hours. A diet of Ralston Purina (St. Louis, MO) mouse chow and water was provided ad libitum, and room temperature was maintained at ~ 21 °C. The Carleton University Committee for Animal Care approved all

experimental procedures and complied with the guidelines set out by the Canadian Council for the Use and Care of Animals in Research.

One week prior to the commencement of the study, baseline home cage activity and sucrose preference measures were carried out as described below. Any animals not displaying an initial preference to the 1% sucrose solution were removed from the experiment (n = 8). Mice (n = 12/group) were then randomly assigned to one of the four experimental conditions (No stress/Saline; No stress/Paraquat; Stress/Saline; Stress/Paraquat).

The study occurred over a 6-week period upon which animals received 3 weeks of chronic unpredictable stress followed by saline or paraquat exposure (2X/week for 3 weeks) which occurred while the chronic unpredictable stress or no stress regimen continued. At the end of the first three-week period (i.e. before the beginning of the injection regimen), stressed animals were subjected to behavioural testing assessing anxiety (i.e. elevated plus maze) and cognitive measures (i.e. working memory performance using the spontaneous alternation behaviour Y maze) against a stressor naïve control group (n = 8), as described below. Motor behaviours (i.e. home cage locomotor activity) and anhedonia (i.e. sucrose preference) were measured at the end of each week throughout the study. Following the 6 weeks of stress or toxin administration, animals received behavioural tests assessing motor, cognitive, anxiety, and depressive-like characteristics at various time points.

At the end of the experimental paradigm, all animals were either rapidly decapitated or transcardially perfused (i.e. four days following last injection and five minutes after the final

behavioural task). All behavioural tests (apart from home cage locomotor activity and sucrose preference testing) were carried out between the hours of 08:00 and 13:00 in order to minimize any diurnal variations.

Chronic unpredictable stress, injection protocol, and behavioural testing

All animals in the experiment were randomly assigned to a 6 week chronic unpredictable stress or a non-stress control group placed in a room separate from stressed mice. Animals in the stress condition received two stressors/day, one in the morning between the hours of 08:00 and 12:00, and one afternoon between the hours of 13:00 and 18:00. Stressors occurred on a variable unpredictable schedule and ranged from mild to severe as per the rationale given by Littlejohn et al. (2014) [302]. The chronic variable stressor regimen included the following stressors: overnight exposure to predator (rat) odor which consisted of placing the mouse in a cage containing soiled bedding and rat feces; lights on during total dark phase (i.e. between 20:00 and 08:00); placement of mouse in an empty polypropylene cages (27 × 21 × 14 cm) and exposing them to 5 minutes of room temperature air from a hair dryer on a low setting; overnight social stress exposure which consisted of placing the mouse in a CD1 retired breeders soiled cage; overnight placement in an empty cage (i.e. void of any nesting or bedding); hanging mouse from tail (1 minute); tilting the mouse's cage at a 30° angle (overnight or up to 4 hours); 5 minute tail pinch which consisted of placing a small insulated clamp over the tail (beginning at the top); 30 minute restraint in triangular plastic bags with a nose-hole for breathing; 30 minute exposure to social stress which consisted of placing the mouse in the cage

of a new aggressive retired breeder CD1 mouse (n = 20; Charles River) until socially defeated (defined as submission or first hostile contact by experimental mouse). Upon submission or first contact, a mesh divider was placed between the two animals for the subsequent duration of the session.

After the first three weeks of the chronic unpredictable stressor procedure, intraperitoneal (i.p) injection of 10 mg/kg paraquat (1,1′-dimethyl-4,4-bipyridinium dichloride; Sigma Aldrich, St Louis, MO, USA) or physiological saline (Sigma Aldrich) commenced. Injections were administered twice a week for 3 weeks on a regular basis between the hours of 08:30 and 09:30. On injection days, during the 30 minutes immediately preceding each injection, the stressed animals were either socially defeated or physically restrained on an alternating fixed schedule. All mice continued to have home cage locomotor activity testing, as well as sucrose preference testing during this period. At the end of the stress and injection period, behavioural tests were conducted for all mice to evaluate signs of: (1) behavioural despair (using the forced swim test), (2) anxiety (using elevated plus maze), (3) cognitive impairment in working memory performance (using the spontaneous alternation behaviour Y-maze), (4) total home cage activity, and (5) movement coordination (i.e. using our rotarod system) as described below.

Home Cage Locomotor Activity

Spontaneous home cage locomotor activity was measured over a complete 12 hour light/dark cycle using our Micromax (MMx) infrared beam-break apparatus (Accuscan Instruments, Columbus, OH, USA) as previously described [303]. Total home cage locomotor

activity is determined based on the number of infrared beam-breaks an animal makes based on 16 infrared wavelengths originating external to the animal's home cage. Following a 30 minute acclimation period in our behavioural testing room post nestlet removal, measurements of home cage locomotor activity occurred once at baseline (Day 0), then again at the end of the 1st (Day 6), 2nd (Day 13), and 3rd (Day 20) week in the pre-injection period. At the beginning of the injection phase of our study, home cage locomotor activity measurements were taken the evening of the 1st (Day 22), 3rd (Day 29) and 6th (Day 40) injection. This behavioural test occurred at least 3 hours following the variable afternoon stressor.

Sucrose Preference Test

In order to assess whether or not paraquat exposure is associated with depressive-like behaviours, and whether or not chronic unpredictable stress alters this behavioural outcome, a modified sucrose preference test was conducted [284], [304]. All animals received sucrose preference training for a period of 5 days upon which they received 2 days of 2% sucrose solution followed by 3 days of a 1% sucrose solution post acclimation. Baseline measurements were taken on the 5th day of training. On baseline and testing days, animals were simultaneously exposed to two 200 ml bottles containing 1% sucrose solution or tap water randomly placed approximately 1cm apart from each other. Amount of solution drank was determined based on bottle weights before and 12 hours after placement (i.e. over a complete 12 hour light/dark cycle). Preference for the sucrose solution was calculated according to the following formula: sucrose intake / (sucrose intake + water intake)*100. In the pre-injection phase of our study (i.e.

the first three weeks), non-stressed and stressed mice received the sucrose preference testing at the end of the 1st (Day 7), 2nd (Day 14) and 3rd (Day 21) week. In the injection phase of our study, animals received sucrose preference testing one day after the 1st (Day 23), 3rd (Day 30), and 6th (Day 41) injection to omit the possibility of any sickness behaviours. This behavioural test occurred at least 3 hours following the variable afternoon stressor.

Spontaneous Alteration Behaviour Y-maze

In order to assess if working memory dysfunction is induced by chronic paraquat exposure, and to assess whether or not chronic unpredictable stress alters this performance, an adapted version of the Y-maze was used, as outlined by Wall and Messier, 2001 [305]. The Y-maze consisted of three arms each 40cm long X 3cm wide enclosed by 13cm high walls which converged on an equilateral triangle at the centre. Each animal was individually placed at random in one of three enclosed arms for a total of 8 min. Alternate arm returns (AAR), same arm returns (SAR), and spontaneous alteration behaviour (SAB) performance was recorded when an animal had placed all 4 paws in the arm runway outside of the centre triangle. SAR's were defined as when an animal left a previously entered arm and then returned to the same arm, with at least 2 paw entry into the centre triangle and without total entry into another arm. AAR was defined as when an animal returned to a previous entered arm after 4 paw entry into another arm (for example arm A to B and then back to arm A). SAB performance was defined when an animal had entered each arm with 4 paws in a sequential order without returning to a previous arm (for example arm A to B followed by arm B to C). For appropriate data analysis,

scores were expressed as percentages, in order to not bias any results affected by total number of arm entries. Thus the following equations were used: %SAR = total number of same arm returns / total number of arm entries X 100, %AAR = alternate arm returns / total arm entries X 100, and %SAB = total number of sequential alternations / total arm entries X 100. The Y-maze was given twice to mice applied to the stressor condition (i.e. once after exposure to three weeks of chronic unpredictable stress and before exposure to saline or paraquat (Day 19), and then again three days following the last injection (Day 42). Performance in this test was assessed only three days following the last injection (Day 42) in animals assigned to the non-stressed condition.

Elevated Plus Maze

In order to measure whether or not paraquat induce anxiety-like behaviour, and whether or not chronic stress alters performance in this task, the elevated plus maze was used, as previously described [306]. The elevated plus maze consists of 4 arms (24.8 cm long X 7cm wide) with two closed arms enclosed by 21 cm high walls and elevated approximately 60cm off the surface of the floor. Each animal was individually placed in the centre of the four arm maze, and behaviour was recorded for a total of 6 minutes using our Any-Maze software, version 4.71. Anxiety-like behaviours measured included the percent time spent in the open versus closed arms during the last 5 minutes of the test. The maze was cleaned with 10% EtOH between trials. Anxiety performance in the elevated plus maze was measured twice in mice applied to the stressor condition (i.e. once after exposure to three weeks of chronic unpredictable stress (Day

21) and then again three days following the last saline or paraquat injection (Day 43). Performance in this test was assessed only on Day 43 in animals assigned to the non-stressed condition.

Rotarod

In order to assess whether or not paraquat exposure affects motor coordination and balance, and whether or not chronic unpredictable stress alters these effects, the rotarod task was used (AccuRotor, AccuScan Instruments, Columbus, OH). Our rotarod protocol consisted of a total of three days in which the animal is exposed to a motorized rod (1cm in diameter) encased in a test chamber (44.5cm x 14cm x 51cm; Accuscan Instruments) a total of three times (spaced one hour apart from each other to minimize fatigue) each day for up to 5 minutes. On the first two days (considered training days) the animal was exposed to a rotating rod which maintained a constant speed (12 rpm for day 1, and 22 rpm for day 2) for a total of 5 minutes whereby they were quickly placed back on the rod when contact with the rotating drum was not maintained. On the third day (considered testing day), the rotating rod increased gradually from 4 to 44 rpm over the 5 minute time span and the time the animal spent on the rotating rod during each trial was measured. In adherence with previous study protocols, the lowest amount time an animal spent on the rotating rod was removed and the remaining times were averaged, presumably to eliminate any accidental slips. The rotarod test was carried out one day after the last injection (Day 41). The chambers were cleaned with 10% EtOH between trials.

Forced Swim Test

The forced swim test (FST) was conducted in order to assess whether the depressive-like effects (i.e. behavioural despair) that are typically induced by chronic unpredictable stress exposure might be similar to those induced by our paraquat dose regimen, and to assess whether or not chronic unpredictable stress alters the altered behaviour provoked by the toxin. A modified version of Porsolt et al. (1977) FST was used whereby mice were individually placed in a glass cylinder 20cm in diameter that contained temperature controlled water (22 ± 1 °C) at a depth of approximately 20cm for a total of 6 minutes. Time immobile was recorded on our camera and scored by an independent observer blind to all experimental conditions. Immediately following the task, animals were dried off and placed in their home cage and quickly transferred to necropsy where rapid decapitation or perfusion was performed. This test occurred four days following the last chronic unpredictable stress or saline or paraquat injection (Day42).

Brain extraction

Five minutes following the final behavioural task (between 8:30am and 11:00am), mice were either rapidly decapitated (n =6/group) or transcardially perfused (n = 6/group) and tissue was collected for western blot or immunostaining respectively. For rapidly decapitated animals, a chilled micro-dissecting block that contained slots (0.5mm apart) for single edged razor blades was used. Brains were quickly excised and the hippocampus was micro-punched from coronal brain sections to assess the effects of paraquat exposure and combined chronic unpredictable stress and paraquat on these regions. The tissue was immediately frozen upon dissection and

stored at -80ºC until processing. In transcardially perfused mice, all animals were given an overdose of sodium pentobarbital and transcardially perfused with 5 ml 0.9% saline (pH 7.2) followed by approximately 45 ml of 4% paraformaldehyde (PFA). Brains were excised and post-fixed at 4ºC for 24 hours in 4% PFA. The following day brains were placed in a 10% sucrose 0.1 M phosphate buffered (PB) solution (pH 7.4) two times 6 hours apart from each other and stored at 4ºC between washes. Following these washes, brains were placed in a 30% sucrose PB solution for 48 hours and then flash frozen at -80ºC until processing.

Immunostaining

Immunohistochemistry was performed to assess whether paraquat, chronic unpredictable stress exposure or the Stress X Paraquat interaction influenced: (1) microglia activation in the SNc, (2) the loss of dopamine fibers in the stratum or (3) dopamine cells in the SNc. As such, markers for microglia (i.e. ionized calcium-binding adapter molecule 1; IBA1; Abcam) and dopamine (i.e. tyrosine hydroxylase; TH; ImmunoStar) were selected. IBA1 is a membrane bound protein specifically expressed on microglia/macrophages and is highly upregulated in activated glial cells [308]. As such, visual expression of the protein can help distinguish between activated and non-activated states [309]. TH is the rate limiting enzyme responsible for dopamine production, and can thus be used as an effective dopamine cell marker in the SNc and striatum respectively [45].

Brains were sliced (40 um thick for striatum; 40um thick for SNc) on our Shandon AS620 cryostat (Fisher Scientific, Ottawa, ON) and sections were immediately placed in a 0.1M PB

solution containing 0.1% sodium azide (pH 7.4). Every second section was selected for each stain (i.e. striatum TH; SNc TH; SNc IBA1). For TH staining, upon tissue processing, slices were washed in a phosphate buffer saline (PBS) (pH 7.4) three times for a period of 5 minutes each, followed by a 30 minute incubation in 0.3% hydrogen peroxide in PBS. Slices were then washed in PBS three times five minutes each and a 1 hour incubation in blocking solution containing 5% normal goat serum (NGS), 0.3% triton-X, with 0.1 M PBS, pH 7.2 commenced. Following removal of the blocker, slices were then incubated in primary antibodies (anti-mouse TH 1:2000; ImmunoStar). The following day, primary antibody removal occurred as sections were washed in PBS three times for a period of 5 minutes each. Following the washes, secondary antibodies were applied to striatum (anti-mouse IgG; 1:500) for a period of 2 hours, and to SNc (anti-mouse HRP; 1:200) sections for a period of 4 hours. Following a three X five minute wash, striatum sections were incubated in HRP (1:1000) for an additional 2 hours. All sections were then washed in PBS three times five minutes each and sequentially exposed to a DAB reaction containing 30% hydrogen peroxide for visualization of HRP. Sections were washed in PBS three times five minutes each following DAB exposure. All sections were then slide mounted and set to dry overnight. Sections were then dehydrated using a series of alcohol and clearene washes and sequentially cover-slipped using DPX. All incubations occurred at room temperature.

For IBA1, upon tissue processing, slices containing the SNc were washed in PBS (pH 7.2) three times for a period of 5 minutes each, followed by a 1 hour incubation in blocking solution containing 5% normal goat serum (NGS), 0.3% triton-X, with 0.1 M PBS (pH 7.2). Following

removal of the blocker, slices were then placed in anti-rabbit IBA1(Abcam) at a dilution of 1:1000 in a primary antibody solution containing 5% NGS, 0.3% Triton X, 0.3% BSA, 94.4% PBS for a period of 2 hours. Sections were then washed in PBS three times for a period of 5 minutes each and then reacted with a secondary Alexa 594 antibody for the appropriate species (i.e. rabbit) at a dilution of 1:1000 in a solution also containing 5% NGS, 0.3% Triton X, 0.3% BSA, 94.4% PBS. In order to get a representative picture of the SNc slices were also stained with anti-mouse TH (1:1000) in the same primary solution followed by a secondary Alexa 350 antibody following appropriate washes. The signals were visualized with immunofluorescence confocal microscopy using Zeiss image acquisition software (Zeiss LSM 510). All slices were the same distance from bregma.

Microglia Activation

State of microglia cell activation of IBA1 sections in the striatum and SNc was rated using a scale described previously [168]. Activation state of microglia was scored on a scale of 0 (non-activated) to 3 (highly activated). A score of 0 was given when all microglia were in their quiescent state upon which these cells have highly ramified processes that are actively surveying the microbial environment of the brain parenchyma. A score of 1 was applied when microglia were in an intermediary (moderate) phase of activation upon which less than 10 cells displayed this state. A score of 2 was applied when the majority of cells were in an active state defined as having an amoeboid like shape with little to no ramified processes. An observer blind to all experimental conditions scored all sections.

Quantification of SNc TH-positive neurons

In order to assess the number of dopamine producing neurons in the SNc, stereological procedures were conducted as previously described [45]. SNc TH+ serial sections were quantified between bregma levels −3.08, -3.16, -3.28, -3.40, and −3.52 by an observer blind to all experimental conditions. Cell counts were conducted using our Micro Bright Field Inc. Stereo Investigator software upon which the entire SNc was outlined under a 2.5x magnification and counts were completed using a 60x oil immersion objective lens.

Quantification of striatal TH-positive neurons

In order to number of TH+ terminals in the striatum, densitometry measures from photopictomicropraphs were used as described previously [45]. In brief, for each striatal section, an area is selected under a 10x magnification and background threshold was determined from images converted to an eight bit format. The total number of white (background) to black (TH+) pixels is determined using Image J software that uses an algorithm used to calculate upper and lower thresholds.

Western blot

Tissue punches collected from hippocampus were used to detect glucocorticoid receptor (GR) and phosphorylated glucocorticoid receptor (pGR) expression, as well as to detect levels of brain derived neurotrophic factor (BDNF; marker of plasticity) in the hippocampus. Indeed, in the current study, we were also interested in paraquats effects outside of the nigrostriatal

system, and in particular to determine if paraquat acts similar to a psychological stressor, in addition to evaluating whether chronic stress influences the effects of the toxin. As such, research from our lab suggested that paraquat can cause upregulation of the stress relevant hormone corticosterone, as well as lead to the disruption of hippocampal functioning by altering BDNF levels [284]. BDNF is a peptide hormone that binds to the tropomyosin-related kinase B (trkB) full length (trkB.FL) and truncated (trkB.T2) receptors and data shows that it can promote the growth, survival, and plasticity of neurons [310]. Importantly reduced levels of this trophic factor in the hippocampus have been related to cognitive impairment, anxiolytic behaviours, and depressive-like characteristics in animal stress models [81]. As such, we thought that given that paraquat can cause a reduction of this stress related factor, paraquat may also cause alterations to other stress related hormones including the GR which is HPA axis mediator. Indeed interactions between BDNF and GR have been shown, and both proteins are downregulated in animals displaying stressor like characteristics, coupled with an increase in inflammatory measures [311]. Accordingly, it was of interest to us to determine levels of the GR in animals exposed to paraquat to determine if paraquat is potentially acting on elements related to HPA axis functioning. Additionally, while we felt it was important to look at total levels of the protein, as reduced availability of the non-activated receptor renders a system to be less responsive to the ligand, we were also interested in evaluating whether paraquat expression leads to altered expression of the activated version. Activation of the receptor can be measured by looking at levels of phosphorylation of the GR at serine 211. Indeed activation of this receptor via corticosterone allows for GR to dissociate from a chaperone complex

containing proteins such as HSP90, HSP70, and FKBP52 and translocate to the nucleus and interact with glucocorticoid response elements (GREs) in DNA and thus influence gene expression [311].

Whole cell lysates from collected from the hippocampus were homogenized in Radio Immuno Precipitation Assay (RIPA) buffer [50 mM Tris (pH 8.0), 150 mM sodium chloride, 0.1% sodium dodecyl sulphate (SDS), 0.5% sodium deoxycholate and 1% Triton X-100] mixed with 1 tablet of Complete Mini ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor (Roche Diagnostics, Laval, QC, Cat #11 836 170 001) per 10 mL of buffer and then sonicated for 10 seconds in ice cold water. The lysed cells were then centrifuged at 5000 RPM with a table top microcentrifuge for 10 minutes at 4˚C. The supernatant was then extracted and protein concentration was determined using bicinchoninic acid (BCA) method (Thermo Scientific). Following protein concentration determination, supernatant was placed in 5x loading buffer (containing (5% glycerol, 5% β-mercaptoethanol, 3% SDS and 0.05% bromophenol blue) and the protein was denatured when placed in a 5 minute heating block at a temperature of 105˚C. Following this step, samples were then placed in a -20˚C freezer until Western blotting commenced.

On day one of analysis, proteins were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE gel (7.5%) containing the separating buffer (370 mM Tris-base (pH 8.8), 3.5 mM SDS), and the stacking buffer (124 mM Tris-base (pH 6.8), 3.5 mM SDS), were placed in running buffer (25 mM Tris-base, 190 mM

glycine, 3.5 mM SDS) and samples, along with the Precision Plus Protein™ Standards Dual Color (Bio-Rad, Hercules, CA, Cat#161-0374), were loaded into the Arcylamide gel (7.5 %) for molecular weight determination at 140 volts. After electrophoresis, proteins were transferred for one hour at 4˚C at 100 volts in transfer buffer solution (25 mM Tris-base, 192 mM Glycine, 20% methanol), onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Cat#162-0177). Thereafter, membranes were dried overnight. The following day, membranes were reactivated using methanol and total protein load concentration was determined.

In order to determine total protein, after a brief methanol rinse (5 seconds), membranes were incubated in REVERT total protein solution for a period of 5 minutes followed by placement into a REVERT wash solution (6.7% Glacial Acetic Acid, 30% Methanol, in water) two times 2 minutes each. Membranes were then quickly rinsed with distilled water and imaged on our LI-COR Odyssey imaging system on the 700 channel for an exposure period of 2 minutes. Membranes were then rinsed immediately post imaging in tris buffered saline ((TBS) pH 7.5 (2 X 5min.)) buffer followed by being blocked with 0.5% fish gelatin (Sigma) in TBS for 90 minutes. Membrane incubation with mouse anti-BDNF (1:1000), rabbit anti-GR primary antibody (1:500; Sigma), rabbit anti-phosphorylated-GR (1:1000; Cell Signaling Technology), for a period of 60 minutes in 0.05% fish gelatin in TBS with 0.1% tween. Any unbound antibody was removed using 15 mL of TBS-T/membrane at room temperature four times five minutes each. Membranes were then incubated for one hour in infrared conjugate directed against the species the primary antibody was raised in (mouse, rabbit, rat 800, LI-COR) at a concentration of 1:20 000 in 0.5% fish gelatin solution containing 0.2% tween and 0.01%SDS. Membranes

were then washed in TBS-T (4 X 5 minutes) followed by 2 X 5 minutes washes in TBS and read on our Licor Odyssy system at the appropriate wavelength for 6 minutes.

Corticosterone assay

In order to test for differences in corticosterone levels an ELISA corticosterone determination assay (Corticosterone #900-097, Lot# D1260724) was performed using trunk blood collected immediately after decapitation. Briefly, all blood samples were collected in EDTA coated Eppendorf tubes, after which they were spun for 20 minutes at 4°C (20 000g). After serum collection, samples were immediately frozen and stored at -80°C. Corticosterone determination was then performed using our SpectraMax microplate reader and quantified.

Data Analysis

All data were analyzed by a 2 (Stress; no stress vs. stress) X2 (Injection; saline vs. paraquat) analysis of variance (ANOVA) followed by Fisher's planned comparisons ($p < 0.05$) where appropriate. When assessing the effects of the first three weeks of chronic unpredictable stress exposure data was analyzed using an Unpaired Student's t Test. Additionally, analysis of the sucrose preference test and total home cage locomotor activity, was completed using appropriate repeated measures ANOVA's conducted with *Time* as the 3rd independent variable followed by the relevant posthoc analysis. All data was analyzed using the statistical software StatView (version 6.0) and and visualized with GraphPad Prism 6 (La Jolla, CA). Differences were considered statistically significant when $p < 0.05$.

Results

Effects of first three weeks of chronic unpredictable stress exposure

Prior to saline or paraquat exposure, mice received three weeks of chronic unpredictable stress and were tested against non-stressed controls in order to verify the ability to the stressor regimen to induce anxiety and cognitive deficits. Hence, these mice were tested using an elevated plus maze and spontaneous alternation behavior Y maze. Accordingly, we report that our three weeks of stress did indeed induce anxiety-like behavior as made evident in the elevated plus maze, wherein stressed mice spent less time in the open arms than non-stressed counterparts ($t = 4.116$, $p < 0.01$; Figure 2 panel A). Furthermore, the chronic unpredictable stress also induced working memory deficits, as stressed mice displayed lower spontaneous alternations than their non- stressed counterparts ($t = 6.534$, $p < 0.01$; Figure 2 panel B). As such, we utilized this stressor regimen for the rest of the study.

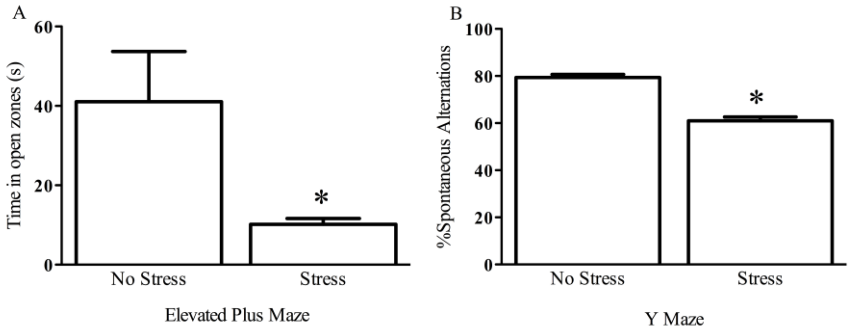

Figure 2: Three weeks of chronic unpredictable stress induces anxiety-like behavior and working memory deficits.

Impact of chronic unpredictable stress on anxiety-like behavior and working memory. The elevated plus maze (panel A) and spontaneous alternation behavior Y-maze (panel B) were used in order to test the effects of three weeks of chronic unpredictable stress on anxiety-like and working memory behaviour respectively. Accordingly stressed mice spent significantly less time in the open zones of the elevated plus maze (panel A) indicating an anxious like state. Stressed mice also displayed working memory deficits as made evident by reduced overall spontaneous alternations (panel B). *$p < 0.05$, relative to non-stressed mice. All data is expressed as mean ± SEM.

Chronic unpredictable stress does not alter home cage locomotor induced by paraquat exposure.

As depicted in Figure 3 panel A, mice did not differ in home cage locomotor activity at baseline prior to receiving stressor treatment or experimental injections. However, while it is clear that all mice had a reduction in home cage locomotor activity over time ($F(6,234) = 34.95$, $p < 0.001$), there was a significant main effect of stress ($F(1,39 = 6.606$, $p < 0.05$) such that stressed mice displayed lower locomotor activity that began after the first two weeks of chronic unpredictable stress exposure and continued until the final test. Additionally, beginning after the third injection, (week 5) non-stressed saline exposed mice had the highest levels of locomotor activity relative to all other groups as a significant Stress X Treatment interaction was observed ($F(1,43) = 3.902$, $p = 0.05$) which was also evident at the 6th injection ($F(1,42) = 10.007$, $p < 0.05$) and confirmed by post hoc comparisons ($p < 0.05$). In other words non-stressed paraquat exposed animals performed similar to their saline and paraquat exposed stressed counterparts and chronic stress did not alter paraquat induced locomotor deficits.

Stress and paraquat treatments impaired motor coordination

The Rotarod measure of coordinated activity revealed a significant Stress X Injection interaction ($F(1, 44) = 8.642$, $p < 0.01$). Indeed, as shown in Figure 3 panel B, the retention time on the rotating drum was significantly reduced by the individual paraquat and stress treatments ($p < 0.05$, relative to controls), but the combination of these two insults resulted in the greatest reduction, such that levels were lower than all other groups ($p < 0.05$).

Paraquat provoked SNc dopamine cell loss which was not altered by chronic unpredictable stress exposure

Four days following the last paraquat injection and stress exposure, mice were sacrificed and a number of surviving TH+ dopamine neurons were assessed within the SNc. Regardless of stress exposure, paraquat alone reduced the number of SNc dopamine neurons ($F(1,16) = 13.584, p < 0.01$). This effect was not further altered in mice that received prior and concomitant stress exposure. (Figure 3 panel C)

Paraquat provoked microglia activation in the SNc which was not altered by chronic unpredictable stress exposure

With regards to SNc microglial morphological state, the ANOVA revealed a significant main effect of paraquat ($F(1,16) = 21.130$, $p < 0.01$), whereby paraquat exposed animals displayed higher microglia activity. Paralleling the TH+ data, stressor exposure had no significant effect. (Figure 3 panel D).

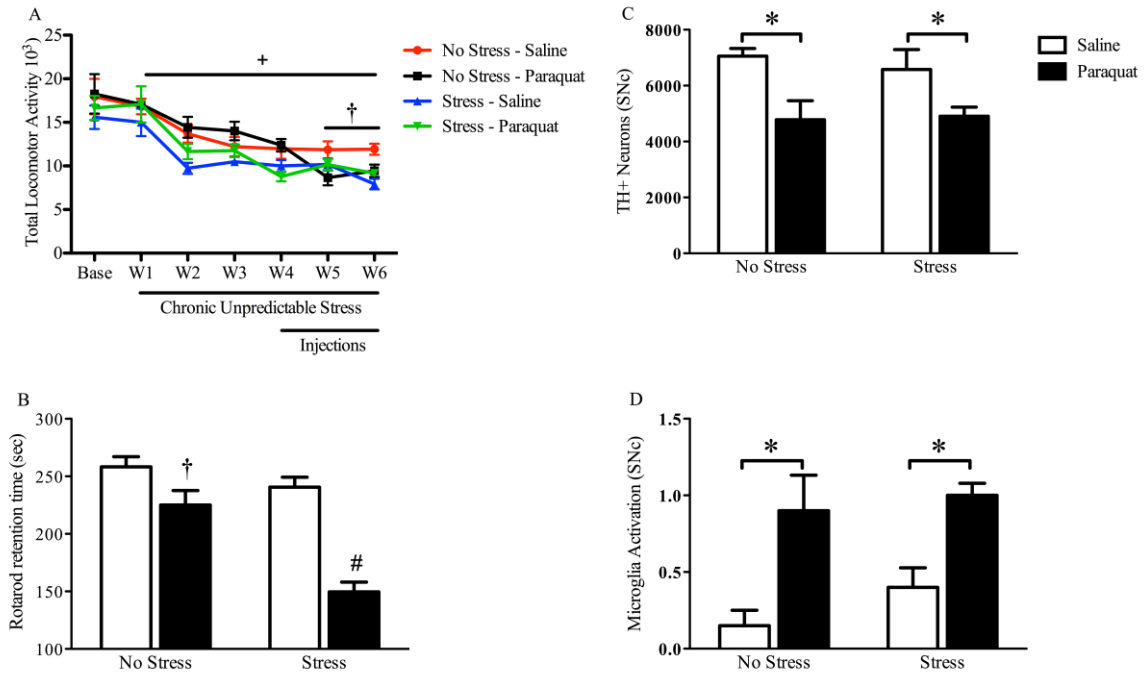

Figure 3: Chronic unpredictable stress enhanced motor impairment but did not alter paraquat provoked SNc neurodegeneration accompanied by regional microglia activation.

Mice were given three weeks of chronic unpredictable stress followed by paraquat (10 mg/kg; i.p.) or saline injections twice/week over three weeks upon which the stressor regimen continued. As shown in panel A, stressed mice had a significant reduction in home cage activity beginning at week 2, which continued until the end of the study. Additionally, paraquat alone provoked a significant reduction in motor activity following the third injection relative to non-stressed counterparts. Panel B shows rotarod retention time, which is a measure of motor coordination. Non-stressed paraquat exposed mice displayed coordination impairment relative to their saline exposed non-stressed counterparts. Moreover, these deficits were enhanced in paraquat treated mice also exposed to the chronic unpredictable stress regimen. However, chronic stress exposure did not alter paraquat induced reduction in number of stereologically counted TH+ SNc dopamine neurons (panel C) or increase activated microglia expression in the region (panel D). * $p < 0.05$, relative to saline treated mice irrespective of stress exposure; + relative to non-stressed mice irrespective of saline or paraquat exposure; # relative to all other groups; † relative to non-stressed saline treated mice. All data is expressed as mean ± SEM.

Chronic unpredictable stress exposure accelerated paraquat induced anhedonia

A sucrose preference test (SPT) was used throughout the 6-week study in order to measure whether or not paraquat-treated animals develop anhedonic symptoms akin to what is expected with chronic unpredictable stress. It was clear that no significant differences were observed in sucrose preference in any of the groups at baseline and during the first four weeks of testing. However results indicate a significant Time X Stress interaction ($F(6,228) = 2.516$, $p < 0.05$) such that, at week 5, stressed animals showed reduced sucrose preference relative to non-stressed animals. However, as evident in Figure 4 panel A, this effect seems to be specific to stressed mice who were also exposed to paraquat ($p < 0.05$). At week 6, stressed animals exposed to saline or paraquat, and non-stressed paraquat animals, showed reduced sucrose preference relative to their non-stressed saline-treated counterparts, and stress did not further exacerbate paraquats effects at this time point ($p < 0.05$).

Chronic unpredictable stress does not alter paraquat induced forced swim deficits

The forced swim test (FST) was administered to assess the amount of time the animals spent immobile, as an indicator of behavioural despair. There was a significant interaction between the stress and paraquat treatments with respect to time spent immobile ($F(1,43) = 6.770$, $p < 0.05$). The follow up comparisons revealed that both the stressor and paraquat treatments alone elevated immobility time relative to non-stressed saline treated controls ($p < 0.05$), however when these two insults were combined there was no further change in immobility (Figure 4 panel B).

Paraquat exposure induces anxiety-like characteristics similar to chronic unpredictable stress exposure in the elevated plus maze

As depicted in Figure 4 panel C, there was a significant Stress X Treatment interaction with regards elevated plus maze performance ($F(1,44) = 5.037$. $p < 0.05$). Specifically, paraquat greatly reduced time spent in the open arms in the non-stressed animals ($p < 0.05$), but had no effect in the stressed mice. However, the stress treatment itself reduced open arm time relative to the non-stressed saline treated controls ($p < 0.05$).

Paraquat exposure induces cognitive-like characteristics similar to chronic unpredictable stress exposure in the spontaneous alternation behavior Y-Maze

In order to assess whether paraquat exposure induced cognitive-like deficits similar to those brought on by chronic unpredictable stress, and to assess whether chronic unpredictable stress exposure impacts paraquats actions, we assessed working memory in the spontaneous alternation behavior (SAB) Y-Maze. Accordingly, as is clear in Figure 4 panel D, a significant interaction was observed ($F(1,41) = 6.406$, $p < 0.05$) such that non-stressed saline exposed mice had a greater number of spontaneous alternations (and thus working memory activity) relative to all other groups ($p < 0.05$). Or alternatively, paraquat induced working memory impairment in this task similar to chronic unpredictable stress and these effects were not altered if paraquat treated mice were also exposed to chronic unpredictable stress regimen.

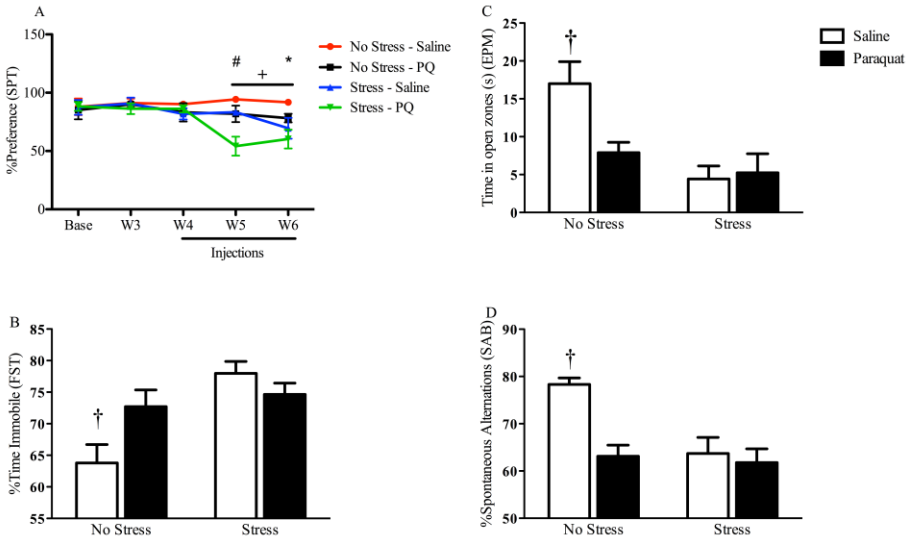

Figure 4: Paraquat provoked behavioral despair, anxiety, and cognitive-like deficits similar to a chronic unpredictable stressor regimen. Chronic unpredictable stress accelerated paraquat induced anhedonia.

Impact of chronic unpredictable stress and paraquat exposure on sucrose preference in the sucrose preference test (SPT; panel A) behavioural despair as measured in the forced swim test (FST; panel B), anxiety in the elevated plus maze (EPM; panel C), and working memory in the spontaneous alternation behaviour Y-Maze (SAB; panel D). As shown in panel A, stressed animals had a reduction in sucrose preference beginning at week 5 (+ $p < 0.05$ relative to non-stressed animals irrespective of saline or paraquat exposure). Paraquat exposed animals also had a reduction in sucrose preference beginning at week 6 (*$p < 0.05$ irrespective of stress exposure), and interestingly this effect seems to be accelerated in stressed paraquat exposed mice as they displayed a reduced preference for the palatable solution beginning at week 5 (# $p < 0.05$ relative to all other groups). As shown in panel B, non-stressed saline-treated animals displayed an increase in mobility time in the FST relative to stressed and paraquat-treated animals († $p < 0.05$). Moreover, in regard to anxiety and cognitive-like behavior, non-stressed paraquat exposed animals displayed deficits similar to stressed animals in the EPM (panel C) and SAB Y-Maze (panel D; † $p < 0.05$). In other words, non-stressed paraquat treated animals performed similarly to stressed mice in the FST, EPM, and SAB and stress did not alter these effects in paraquat exposed animals. All data is expressed as mean ± SEM.

Paraquat increases plasma corticosterone concentrations similar to a chronic unpredictable stress regimen

As shown in Figure 5 panel A, paraquat treatment influenced plasma corticosterone levels ($F(1,20) = 26.052$, $p < 0.0001$), such that paraquat exposed mice had elevated HPA activity regardless of stress exposure. Stressed animals also displayed elevated corticosterone levels irrespective of paraquat exposure ($F(1,20) = 5.896$, $p < 0.05$). However stress did not alter paraquat induced corticosterone activity.

Paraquat and chronic unpredictable stress exposure alters hippocampal BDNF levels

Disturbances in hippocampal plasticity mediated by neurotrophic factors may be important for the co-morbid neuropsychiatric symptoms (i.e. anxiety and depression) as well as the cognitive deficits observed in the disease, and may provide further validation that paraquat behaves similar to a chronic stress exposure which typically results in trophic factor reduction. To this end, we tested hippocampal levels of the neurotrophin BDNF. We found that stress ($F(1,16) = 14.519$, $p < 0.05$) irrespective of paraquat exposure and paraquat exposure ($F(1,16) = 11.280$, $p < 0.05$) irrespective of stress exposure significantly reduced BDNF levels relative to saline or non-stress treated mice respectively (Figure 5 panel C). Likewise, we also found a significant interaction ($F(1,16) = 17.949$, $p < 0.05$) such that saline treated non-stressed mice had higher levels of BDNF relative to all other groups however stress exposure did not further alter the paraquat driven reduction in the BDNF levels.

Chronic unpredictable stress reverses paraquat reduction of hippocampal GR levels

A significant interaction was observed in regards to total GR level ($F(1,12) = 13.287$, $p < 0.0034$). Specifically, as shown in Figure 5 panel B, and confirmed by follow-up comparisons, paraquat reduced hippocampal GR levels in non-stressed mice, relative to saline or stressed-paraquat treated counterparts ($p < 0.05$). These effects were similar to those in saline treated stressed mice as they also displayed reduced hippocampal GR levels in comparison to their non-stressed saline or stressed- paraquat treated counterparts ($p < 0.05$). No effects were observed in regard to pGR expression (Figure 5 panel D).

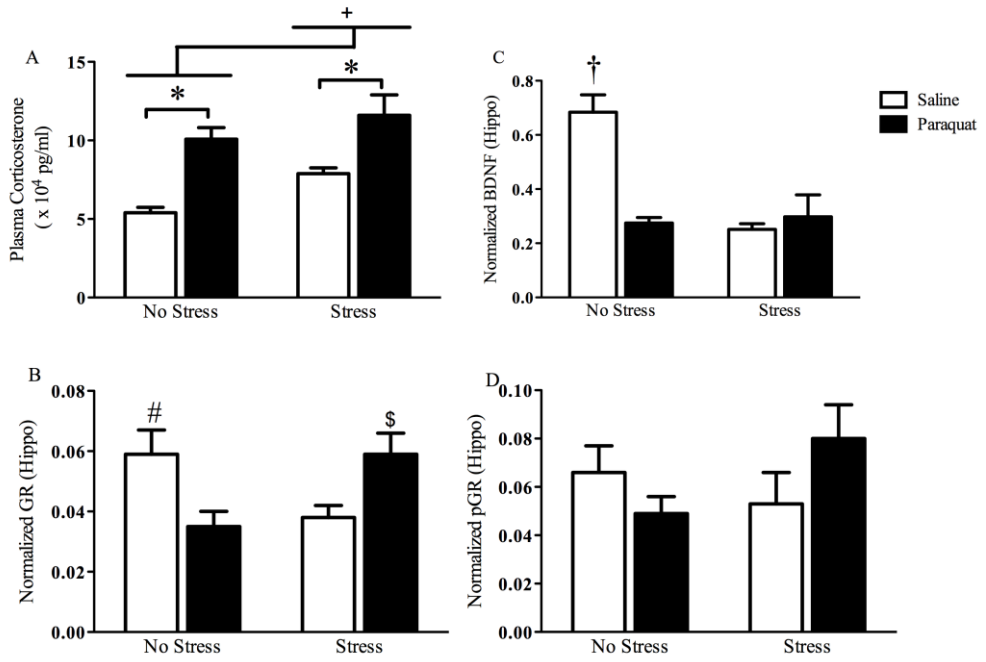

Figure 5: Paraquat provoked HPA activity alterations similar to a chronic unpredictable stressor regimen. Chronic unpredictable stress reverses the GR deficits in mice also exposed to the toxin.

Effects of paraquat administration on plasma corticosterone and hippocampal brain derived neurotrophic factor (BDNF) and glucocorticoid receptor (GR) levels. As shown in panel A, paraquat and chronic unpredictable stress exposure significantly increased plasma corticosterone levels which was not altered in paraquat animals also exposed to the chronic unpredictable stress regimen (panel A). In regard to BDNF levels, non-stressed paraquat exposed animals had a reduction in expression of the trophic factor similar to animals exposed to chronic unpredictable stress, however paraquat did not alter these effects in stressed mice (panel C). At the same time, paraquat exposure or stress exposure did not increase levels of the active form of GR (pGR; panel D). The toxicant dose regimen did however reduce GR levels in non-stressed mice similar to saline exposed mice exposed to our chronic unpredictable stress regimen, which was reversed in our stressed paraquat exposed mice (panel B) $*p < 0.05$ relative to saline treated mice irrespective of stress exposure; + $p < 0.05$ relative to non-stressed mice irrespective of paraquat exposure; † $p < 0.05$ relative to all other groups; # and $ $p < 0.05$ relative to non-stressed paraquat treated mice and saline treated mice exposed to chronic unpredictable stress. All data is expressed as mean ± SEM.

Discussion

It was reported that the widespread brain alterations induced by chronic psychological stress may exacerbate the motor and non-motor behavioral features of PD [77]. However, whether psychological stress impacts the motor and select non-motor behaviors (i.e. cognitive and neuropsychiatric symptoms) and neurological deficits induced in PD animal models involving paraquat has yet to be fully examined. Hence, this was a focus of the present study. In contrast to our hypothesis that exposure to a chronic stress regimen would alter the motor and non-motor behavioral features of PD, paraquat and the stressor exposure generally did not interact to augment behavioral or biological outcomes assessed. Two exceptions however include the observance that chronic unpredictable stress potentially accelerated anhedonia in a sucrose preference test in mice exposed to the toxin, and the observance that motor performance on the Rotarod test was greatly reduced in the paraquat and chronic stress combination. This will be discussed further in the ensuing sections. Most interesting however, paraquat itself had many effects that were similar to that of the stressor treatment, which of course is consistent with our proposition that the pesticide can act as a systemic stressor.

Although scant, there are some previous data showing that paraquat can induce cognitive, emotional, or affective behaviours in mice [331, 332], with the present data greatly extend these findings. Paraquat has been shown to accumulate in areas outside of the motor system including those highly implicated in cognitive and neuropsychiatric disturbances [295], [313], thereby giving it ready access to influence such processes. Moreover, the toxin has been shown to

produce alterations in neurotransmitter activity in areas extending throughout the brain including the prefrontal cortex, hippocampus, locus coeruleus, and hypothalamus [314], [315]. Similarly, oxidative and inflammatory effects induced by the toxin have been observed in frontal, limbic, and brain stem regions [45], [314], [315]. and it has been demonstrated that exposure can negatively affect neuroplasticity, in parallel with a variety of behavioral disturbances [285], [291], [292], [315]–[318]. In fact, ability of the toxin to induce cell death in brainstem and midbrain structures including the locus coeruleus and ventral tegmental area (VTA) respectively has been shown with exposure to relatively high doses [319], [320].

In combination with the findings from our current study, these results provide support that exposure to environmental pesticides, including paraquat, have a capacity to contribute to the development of non-motor symptoms often evident in PD through altering brain processes outside of the nigrostriatal system, in addition to contributing to the cardinal motor deficits. Indeed, in our current study, we found that paraquat induced depressive measures (i.e. anhedonia, behavioral despair/learned helplessness), along with provoking working memory deficits/cognitive impairment, and anxiety-like behaviour as measured in the sucrose preference test, forced swim test, spontaneous alternation behavior Y maze task, and the elevated plus maze, respectively. Moreover paraquat exposure altered hippocampal expression of the neuroplastic factor BDNF, as well as the stress relevant glucocorticoid receptor (GR). Yet, it should be underscored that while our current behavioral findings are in agreement with some previous studies [284], [293] they are inconsistent with previous findings by Campos et al., 2013 [312]. Indeed, in this study, it was found that while paraquat exposure did induce

behavioral despair in the forced swim test, it did not provoke behavioural signs of anhedonia or cognitive impairment [312]. Perhaps however the differences observed were due to treatment administration, age upon exposure, or inherent species differences, as in the aforementioned study, paraquat was given to aged rats via osmotic minipump as opposed to mice administered the toxin into the cavity in the current study.

Our present findings demonstrating that paraquat reduced BDNF in the hippocampus is consistent with the idea that neuroplastic changes may drive cognitive, anxiety, and depressive-like behaviors [321]. Indeed, psychological stressors were previously noted to act in this manner, and conversely, the administration of the trophic factors, such as BDNF, reversed the neuropsychiatric and cognitive effects induced by a number of stressors [322], [323]. In addition, the chronic stressor treatment alone as expected, was found to also provoke a reduction in hippocampal BDNF and had behavioral consequences [324]. However, as already noted, there were no synergistic or even additive effects with paraquat and stress co-administration with regards to BDNF levels.

Beyond the BDNF changes, paraquat also activated the HPA axis in a manner similar to that of more traditional stressors, which could explain some of our behavioral findings. In fact, we found that paraquat alone actually increased levels of corticosterone to a greater extent than did the current chronic stressor exposure. When looking at these insults together, there was a slight additive effect but it was clearly apparent that these two insults do not synergize in their ability to stimulate HPA activity. It would not at all be surprising that paraquat and chronic

unpredictable stress activate the HPA axis through different mechanisms. At the very least, as first mentioned by Herman and Cullinan (1997), the systemic stress of paraquat is acting more directly on the hypothalamus, whereas the psychological elements of the chronic stressor involved higher order interpretation via the cortex and limbic loops [325]. Another element that should be considered is the fact that it could be the distress caused by paraquat that is activating the HPA axis or alternatively, it might be the oxidative-inflammatory stress known to be induced with the brain that gives rise to this effect [326]. Oxidative stress factors including superoxide and nitric oxide, known to be provoked by paraquat, have been associated with HPA activation [327]. Conversely, such factors are not induced by most psychological stressors unless they are particularly severe and protracted [328], [329]. Indeed, the exceedingly long half-life of paraquat in the brain (~28 days), compared to the periphery (8-36 hrs) would give the compound ample time to cumulatively activate stress circuitry [295].

In parallel with the corticoid changes, hippocampal glucocorticoid receptor (GR) expression was altered by the two insults. In this regard, both the stressor and paraquat markedly reduced GR levels, but curiously, their combination actually cancelled out this effect. This surprising finding is hard to reconcile. But may be related to the ability of the stressor treatment to alter paraquat metabolism, such that the pesticide's accumulation in the brain is somehow limited. It will be recalled that the stressor regimen commenced 3 weeks prior to the first paraquat injection and this may have resulted in compensatory responses that blunt the impact of paraquat. Of course, from an alternate view, perhaps the paraquat treatment also up-regulated signaling pathways that were antagonist or competed with the stressor effects. In fact, we

previously found a somewhat similar interaction between paraquat and age [284]. In this case, low dose paraquat treatment (1/10th of the present does) increased the phosphorylated form of GR within the hippocampus, and simply aging alone (17 months of age) had a similar effect. Curiously, however, when paraquat was combined with this excessive age the individual impact of the two insults was absent [284].

Intriguingly, in regard to the non-motor behaviors, while stress did not appear to alter the paraquat induced anxiolytic or cognitive impairments in the current study, the anhedonic response was modestly accelerated towards the end (i.e. 5th and 6th weeks) of the experiment when the pesticide exposure overlapped with that of the stressor. However, the paraquat alone, stress alone, and paraquat + stress induced reduction in preference was limited, and the fact that stress alone didn't alter sucrose preference until near the end of the experiment represents a caveat in our study. That said, one study did report that a psychologically-relevant stressor rendered mice more vulnerable to the non-motor effects of MPTP ([102]). Likewise, our own previous work demonstrated that acute mild stress (but fairly weaker than the current procedure) together with paraquat induced sucrose preference deficits to a greater extent than did each of the individual insults in younger mice [283].

In addition to altering anhedonia, the present study revealed for the first time that paraquat and a psychological stressor acted in a synergistic fashion to impair motor coordination, as revealed using a Rotarod test. This is important given the obvious psychological stress that many PD patients undoubtedly experience in response to diagnosis and progression of the

disease. In fact, tremor severity has been known to be increased during times of psychological distress, in human PD patients [298]. However, the mechanisms responsible for the augmented Rotarod deficit presently observed are not clear. Indeed, we did not find any augmented neuronal loss or microglial activation with the stressor + paraquat combination. However, we did not assess neurotransmitter levels, which clearly could be linked to the deficit. Indeed it has been demonstrated that paraquat can alter striatal levels of dopamine [295], and it was previous found that performance in the task is dependent on an intact nigrostriatal dopamine system [330], [331]. Moreover, motor coordination deficits improve following treatment with antiparkinsonian agents including levodopa [331]. However it is highly possible that other neurochemical alterations in motor learning circuit gave rise to the ability of the stressor to augment paraquats impact [330].

Conclusions: Results from the current study showed that there was mostly no additive or synergistic interaction between chronic unpredictable stress exposure and paraquat on motor and most non-motor behavioral outcomes (with the exception of the Rotarod and weeks 5 and 6 for sucrose preference). Most importantly, our study did support the notion that paraquat exposure behaves as a systemic stressor, with regards to HPA activity and hippocampal processes, as well as behavioral outcomes. Hence, it is clear that such toxicant exposure can contribute to not only the primary motor but also the wide range of comorbid aspects of PD.

Chapter 2: Leucine rich repeat kinase-2 (LRRK2) modulates microglial phenotype and dopaminergic neurodegeneration.

Highlights

- Global ablation of LRRK2 protected against LPS + paraquat induced dopamine cell death in the substantia nigra.

- LRRK2 ablation inhibited LPS-induced microglia activation in the substantia nigra.

- LRRK2 ablation diminishes the LPS induction of phagocytic regulator WAVE2.

Abstract

Leucine rich repeat kinase 2 (LRRK2) is a common gene implicated in Parkinson's disease (PD) and many inflammatory processes. Thus, we assessed the role of LRRK2 in the context of endotoxin (LPS) induced inflammation of the substantia nigra and the effects of the environmental toxicant, paraquat that has been implicated in PD. Here we found that LRRK2 ablation provoked a phenotypic shift in LPS primed microglia, with their "activated" morphology and up-regulation of inflammatory phagocytic regulator, WASP-family verproline homologous protein-2 (WAVE2; critical for actin remodeling), being diminished while the anti-inflammatory chemokine receptor, chemokine receptor 1 (CX3CR1), was elevated in isolated microglia. Most importantly, LRRK2 ablation prevented the loss of dopaminergic neurons and motor behavioral deficits induced by LPS priming followed by paraquat exposure. We are the first to show the importance of LRRK2 in multi-hit toxin model of PD. These data are consistent with the proposition that LRRK2 is a specific mediator of WAVE2-associated inflammatory processes which impact neurodegeneration.

Significance: It is clear that inflammatory processes are linked to PD and that these may be triggered by environmental threats. Here, we demonstrate the importance of the LRRK2 gene as a general regulator of brain and peripheral inflammatory processes relevant for PD. We show that LRRK2 deficiency prevents the loss of dopamine neurons and motor impairment induced by environmental endotoxin and toxicant exposure. In particular, LRRK2 was critical for modulating microglial state through the actin reorganization protein, WAVE2. These data show

the importance for LRRK2 in the coordination of brain and immune system responding to environmental challenges that have been linked to PD and its potential comorbid symptoms.

Introduction

Epidemiological and experimental studies have implicated environmental and inflammatory insults in Parkinson's disease (PD) [332]–[334]. For instance, environmental toxicants, such as the commonly used pesticide paraquat, has been implicated in disease development by numerous reports [34], [277], [286], [335]. Indeed, increased incidence of PD has been associated with exposure to the herbicide, and this effect was even more pronounced among individuals that also possessed risk alleles for PD [26], [336]. Among the alleles implicated in PD, polymorphisms in the gene, leucine rich repeat kinase 2 (LRRK2), are the most common genetic changes observed in the disease [3]. LRRK2 is a multi-domain complex protein characterized by protein kinase and GTPase domains [337], [338]. Although mutations in LRRK2 can cause an autosomal dominant form of the disease, incomplete penetrance (~30–70%) of these mutations suggests a role for environmental and/or immune factors as "triggers" [29]. Besides being detected in the kidney, thymus, lung, and lymph nodes, low levels of LRRK2 mRNA and protein were found in midbrain dopamine neurons, as well as in the striatum, cerebral cortex and hippocampus [339], [340]. However, recent studies demonstrated that the highest levels of LRRK2 occurred in immune cells, including circulating B lymphocytes, dendritic cells and macrophages [259], [261].

Consistent with a multi-hit hypothesis for PD [34], [333], [334], [341], previous findings from our lab demonstrated that LPS infusion into the substantia nigra pars compacta (SNc) sensitized the microglial response to later exposure to paraquat and this resulted in an enhanced

neurodegenerative response [46]. While the mediators of microglial reactivity to immune priming are not clear, we hypothesize that LRRK2 plays a critical role favoring an enhanced inflammatory state. Many factors determine the particular "activation" state of phagocytic cells, such as microglia and macrophages, but among these, CX3CR1 and WAVE2, may be particularly important [342]–[344]. Indeed, the microglial bound CX3CR1 fractalkine chemokine receptor exerts robust anti-inflammatory actions [345], and in fact, CX3CR1 was recently implicated as a negative regulator of microglial dynamics in response to pathological a-synuclein insult [346]. Conversely, WAVE2 is a regulator of the actin remodeling required to facilitate motility, along with pro-inflammatory and phagocytotic states [347], [348]. In fact, members of our group recently identified WAVE2 as a novel interacting partner with LRRK2 and that it is critical for the phagocytosis effects of macrophages [270]. So, CX3CR1 and WAVE2 may be critical for shifting inflammatory states of central and peripheral phagocytic cells.

Presently, we sought to evaluate whether LRRK2 knockout would influence the ability of intra-SNc LPS infusion to prime microglia and influence the neurodegenerative and functional effects of later paraquat exposure. Our data did indeed indicate that knocking out LRRK2 prevented the loss of SNc dopamine neurons and motor impairment in LPS primed mice that later received paraquat. LRRK2 ablation caused a phenotypic shift (with reduced WAVE2 and increased CX3CR1) in isolated SNc microglia primed with LPS. Thus, it appears that LRRK2 is an important regulator of LPS and paraquat provoked central effects that characterize PD.

Materials and Methods

LRRK2 KO and WT animals

Establishment of the LRRK2 -/- knockout (KO) mouse, which develop normally and display a functionally intact dopaminergic system, has been previously described [349]. LRRK2 KO and wild type (WT; LRRK2 +/+) mice used in this study were raised on a C57BL/6*J* genetic background and obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were interbred for several generations in our lab and backcrossed every tenth generation in order to prevent genetic drift. LRRK2 KO mice and littermate controls used in the study were obtained via a heterozygous (-/+) X heterozygous mutant (-/+) breeding scheme. Genetic backgrounds were confirmed through PCR analysis (as described below). The Carleton University Committee for Animal Care approved all experimental procedures and complied with the guidelines set out by the Canadian Council for the Use and Care of Animals in Research.

Genotyping

DNA was extracted from lysed tail snips (0.5-1 cm long) using the DNeasy DNA purification system (Quigen). Following purification, DNA concentrations were determined using the NanoDrop 1000 spectrophotometer (Thermo Scientific). A total of 2ul of diluted DNA was then added to a master mix solution containing ddH_20 (2.1 µL), 25 mM $MgCl_2$ (0.96 µl), 5x KAPA LR HotStart Buffer (2.40µl), 5mM DNA loading dye (1.66 µL), 2.5mM dNTP

(0.96 µL2.5 U/ µl KAPA LR HotStart Taq (0.12 µl), 20 uM 12725 forward primer (0.6 µl), 20 uM 12726 reverse primer (0.6 µl), and 20 uM 12838 WT reverse primer (0.60 µl) (Jackson Laboratory). DNA amplification was conducted using our PTC-200 thermo cycler (MJ Research). Forward, reverse, and WT reverse primer (Invitrogen) sequences are listed below:

Primer Type	**Sequence (5' → 3')**
Forward	CTA CCA GGC TTG ATG CTT TA
Reverse	TCT GTG ACA GGC TAT ATC TC
WT Reverse	CAG TAC TGT GCG ATC CCG TA

Animal genotype was determined via gel electrophoresis following DNA amplification. Amplified DNA containing 3.3μl of Cyan/Orange Loading Dye (Invitrogen) was added to a 2% agarose gel containing Syber Green II dye (Invitrogen). DNA was run at 130V on our BioRad PowerPac™ Universal Power Supply for approximately two hours. Subsequently, the gels were viewed on the BioRad UV camera and genotypes were assigned accordingly.

Study 1: LPS priming with later paraquat exposure: Effects of LRRK2 KO

General Experimental Design

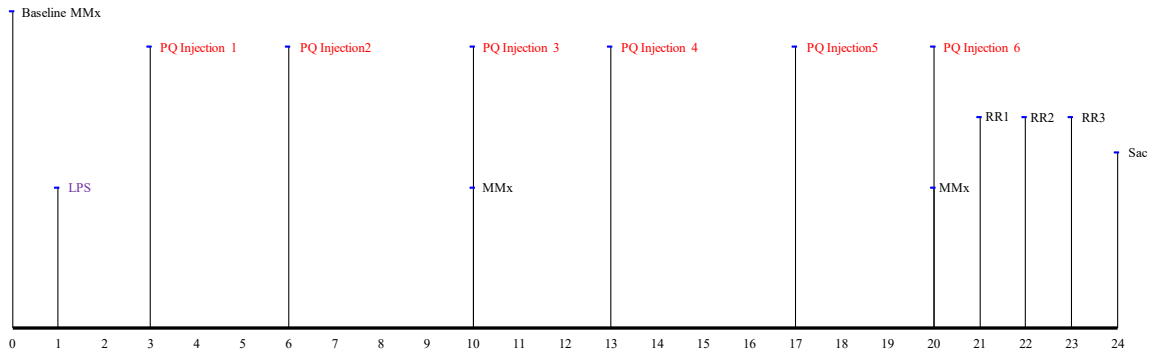

Figure 1: Schematic timeline of study 1

Schematic timeline for study 1. (LPS = lipopolysaccharide; MMx = micromax PQ = paraquat; RR = rotarod)

A schematic timeline for the study 1 can be observed in Figure 1. Ninety-six male LRRK2 KO or WT littermate controls aged 3 months were obtained from our in-house breeding colony. All animals were singly housed at 2 months of age in standard polypropylene cages (27 × 21 × 14 cm) and maintained on a 12-h light/dark cycle with lights on at 08:00 hours. A diet of Ralston Purina (St. Louis, MO) mouse chow and water was provided ad libitum, and room temperature was maintained at ~ 21 °C. Mice (n = 10-12/group) were randomly assigned to one of eight experimental conditions (WT/Saline/Saline; WT/Saline/Paraquat; WT/LPS/Saline; WT/LPS/Paraquat; KO/Saline/Saline; KO/Saline/Paraquat; KO/LPS/Saline; KO/LPS/Paraquat).

Following baseline measurements, all mice were stereotaxically injected with vehicle (saline) or LPS and two days later began a chronic saline or paraquat i.p. injection regimen (twice/week for 3 weeks). Home cage motor activity and motor coordination were assessed to determine impact of LRRK2 on motor function. Home cage locomotor activity was assessed once at baseline and then on the evening of the final injection (Figure 1). Motor coordination was assessed three day following the final injection (Figure 1). At the end of the experiment, all animals were transcardially perfused four days following the final paraquat or saline injection.

Central LPS exposure and paraquat injection regimen

LRRK2 KO and WT littermate controls were stereotaxically injected with either 0.9% saline (2µg) or LPS (2µg; Sigma-Aldrich) directly above the SNc in the left hemisphere (with respect to bregma: anterior–posterior −3.16 mm, ± lateral 1.2 mm, ventral −4.0 mm). Prior to stereotaxic injection all mice were anaesthetized using variable flow oxygen-rich isoflurane inhalation. Animals were then given a subcutaneous tramadol injection to treat any pain incurred by the surgical procedures. The area involving surgical cut was shaved and topical iodine was applied before incision. A Kopf insarea of incision, instruments Model 940 stereotaxic frame was used for the surgical procedure. Infusions were performed using a Harvard Apparatus Pico Plus syringe pump whereby 2µl solution was administered at a flow rate of 0.4 µl/min through polyethylene tubing attached to a Hamilton 25µl syringe with a 22-gauge needle. Following infusion, the L-shaped PlasticOne 328OP Osmotic Pump cannula was

held in position for an additional 5 minutes to ensure the infused solution had an appropriate time to diffuse prior to retraction of the cannula. Once removed the injection site was covered with BoneWax® and the incision was a stitched close. A topical anesthetic jelly containing 2% lidocaine hydrochloride (Xylocaine, AstraZeneca) and animals were then placed on a heating pad for 30 minutes. One day following stereotaxic injection, animals were given subcutaneous tramadol twice spaced 6 hours apart from each other to alleviate any post-surgical pain.

Two days following LPS exposure animals received intraperitoneal (i.p) treatment with paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride; Sigma Aldrich, 10mg/kg) or an equivalent volume of physiological saline (Sigma) 2 times per week for 3 consecutive weeks. All injections occurred in the morning beginning at 8:30am.

Home cage Locomotor Activity

Spontaneous home cage locomotor activity assessment was completed over a complete 12 hour dark cycle using our Micromax (MMx) infrared beam-break apparatus (Accuscan Instruments, Columbus, OH, USA) as described in previous studied conducted by our lab [291]. Following a 30-minute acclimation period in our behavioural testing room post nestlet removal, measurements of home cage locomotor activity occurred once at baseline (Day 0), then again on the evening of the 6^{th} (Day 20) injection.

Rotarod

In order to assess motor coordination and balance deficits, a rotarod (AccuScan Instruments, Columbus,) task was used, in which the ability to maintain contact with a rotating drum is determined. All animals were exposed to test chambers (44.5cm x 14cm x 51cm; Accuscan Instruments) consisting of a motorized rod (1cm in diameter) for three consecutive days. Training occurred on the first two days of rotarod testing. On Day 1 of training, animals were exposed to a rotating rod moving at a constant speed of 12 rpm for a total of five minutes three times spaced one hour apart from each other to minimize any fatigue. Day 2 consisted of the same procedure, however motorized speed of bar was increased to 22 rpm. On testing day, animals were placed on an accelerating rod three separate times (spaced 1 hour apart from eachother) that increased gradually from 4 to 44 rpm and motor coordination was measured. Motor coordination was defined as the average of the longest two times (out of three) an animal spent on the rotating rod before falling off or reaching the end of the 5-minute trial.

Immunostaining

Four days following the last saline or paraquat injection, mouse brains were prepared for immunohistochemical assessment of microglial state and number of surviving dopamine neurons in the SNc. Accordingly, markers for microglia (i.e. CD68) and dopamine (i.e. tyrosine hydroxylase; TH) were assessed. On day 24 of the experiment (between 8:30am and 11:00am), all animals were given an overdose of sodium pentobarbital and transcardially perfused with 5 ml 0.9% saline (pH 7.2) followed by approximately 45 ml of 4% paraformaldehyde (PFA).

Brains were excised and post-fixed at 4ºC for 24 hours in 4% PFA. The following day brains were placed in a 10% sucrose 0.1 M phosphate buffered (PB) solution (pH 7.4) two times 6 hours apart from each other and stored at 4ºC between washes. Following these washes, brains were placed in a 30% sucrose PB solution for 48 hours and then flash frozen at -80ºC until processing.

Brains were cut (40um) and sections were immediately placed in a 0.1M phosphate buffer (PB) solution containing 0.1% sodium azide (pH 7.4). Every second section was selected for tissue processing. Sections were washed in a phosphate buffer saline (PBS) (pH 7.4) three times for a period of 5 minutes each, followed by a 30-minute incubation in 0.3% hydrogen peroxide in PBS. Sections were then washed in PBS three times five minutes each and a 1-hour incubation in blocking solution containing 5% normal goat serum (NGS), 0.3% triton-X, with 0.1 M PBS, pH 7.2 commenced. Following removal of the blocker, sections were then incubated in primary antibodies (anti-mouse TH 1:2000 or anti-rat CD68 1:2000; ImmunoStar and Serotec respectively). The following day, the wash procedures were repeated followed by incubation with the appropriate secondary antibodies. Sections were then washed in PBS three times five minutes each and sequentially exposed to a DAB reaction containing 30% hydrogen peroxide for visualization of HRP. All sections were slide mounted, dehydrated and then cover-slipped using DPX.

Microglia Quantification

State of microglia activation in the SNc was rated using a scale previously developed in our lab [168]. Between bregma levels −3.08, -3.16, -3.28, -3.40, and −3.52 of the SNc under 20x magnification using our MicroBrightField Inc. Stereoinvestigator software, the entire SNc was outlined and activation state of microglia was scored on a scale of 0 (non-activated) to 3 (highly activated). Microglia are given a score of 0 when they display highly ramified thin processes indicative of a quiescent state upon which these cells have highly ramified processes that are actively surveying the microbial environment of the brain parenchyma. When less than ~20% or ~50% of the microglia are characterized by having a thick amoeboid shaped soma with shortened thick branches a score of 1 or 2 is given respectively. Finally when the majority of cells were in an active state characterized as having the amoeboid shape and little to no branches a score of 3 was applied. An observer blind to all experimental conditions scored all sections.

Quantification of SNc TH-positive neurons

In order to assess the number of dopamine neurons in the SNc, stereological procedures were conducted as previously described [45], [168]. Briefly, between bregma levels −3.08, -3.16, -3.28, -3.40, and −3.52 SNc TH+ serial sections were quantified by an observer blind to all experimental conditions. Cell counts were conducted using our MicroBrightField Inc. Stereoinvestigator software upon which the entire SNc was outlined under a 2.5x magnification and counts were completed using a 60x oil immersion objective lens.

Quantification of striatal TH-positive neurons

In order to number of TH+ terminals in the striatum, densitometry measures from photopictomicropraphs were used as described previously [45]. In brief, for each striatal section, an area is selected under a 10x magnification and background threshold was determined from images converted to an eight bit format. The total number of white (background) to black (TH+) pixels is determined using Image J software that uses an algorithm used to calculate upper and lower thresholds.

Data Analysis

All data for study 1 were analyzed using 2 (Genotype; WT vs. KO) X 2 (Primer; saline vs. LPS) X2 (Injection; saline vs. paraquat) ANOVAs followed by Fisher's planned comparisons ($p < 0.05$) where appropriate. Additionally, analysis of home cage locomotor activity, was completed using appropriate repeated measures ANOVA's conducted with *Time* as the 3rd independent variable followed by the relevant posthoc analysis. All data was analyzed using the statistical software StatView (version 6.0) and differences were considered statistically significant when $p < 0.05$.

Study 2: LRRK2-related mechanisms of LPS priming of SNc microglia

Our findings from study 1 (displayed below) demonstrated that LRRK2 may be involved in the microglia mediated degeneration processes of SNc dopamine neurons that is induced by

LPS exposure followed by treatment with paraquat. That is, we found that global LRRK2 ablation protected against the LPS + paraquat provoked microglia activation that accompanies the loss of dopamine neurons and motor behavioral deficits. As such it was of interest to us to assess the mediators of microglial reactivity to the LPS immune priming as they are not clear. Accordingly, this study sought to evaluate the state of microglia in the SNc following priming (i.e. 2 days after LPS infusion). Thus, the neuroinflammatory environment in the SNc (and whether LRRK2 KO modifies it) at the time when the first paraquat injection would commence following LPS exposure was assessed.

General experimental paradigm

At 3 months of age, male WT or LRRK2 KO mice bred in house were randomly assigned to one of four experimental conditions (WT/Saline; WT/LPS; KO/Saline; KO/LPS). All mice (N = 68) were stereotaxically infused with saline or LPS directly above the SNc (as described earlier). Two days following the infusion, mice were either rapidly decapitated (n =6/group) or transcardially perfused (n = 6/group) for western blot in whole tissue, or for immunostaining. An additional 24 animals were used (n = 4-6/group) for microglia isolation and collection for subsequent western blot analysis.

Brain extraction

For rapidly decapitated animals, a chilled microdissecting block that contained slots (0.5mm apart) for single edged razor blades was used. Brains were quickly excised and sections were micro-punched from coronal brain sections. The tissue was immediately frozen upon dissection and stored at -80ºC until processing. The areas dissected/of interest reported for this study included the SNc for both microglia isolation and whole tissue analyses. Brain extraction in transcardially perfused mice occurred in the exact same manner as described in study 1.

Microglia isolation

For microglia isolation, column-free magnetic separation was performed as previously described [350]. Using a EasySep mouse CD11b+ (microglia marker; Serotec) isolation kit (STEMCELL Technologies) SNc cells were dissociated by adding 2.5ml of trypsin (0.25% in PBS) in a 5mL tube and incubating at 37 ºC for 10 mins with gentle shaking and the reaction was stopped using 3mL of DMEM. To get a single cell suspension, cells were then passed through a strainer. Remaining cells were then centrifuged for a period of 10 minutes (1200 RPM) and supernatant was removed upon which cells were resuspended in an HBSS solution. Cells were then counted on a hemocytometer. Following determination of number, cells were then resuspended at a density of $1X10^8$ cells/ml (between 0.1 and 2.5mls) in a 5ml glass tube and 50ul/ml of the labelling reagent CD11b-Phycoerythrin (CD11b-PE with anti-CD16/32-an FcR blocker) was added and incubation occurred for a total of 15 minutes at room temperature. Following incubation, 70ul/ml of selection cocktail was added to the sample and further

incubated for an additional 15 minutes. 50ul/ml of dextran coated EasySep magnetic beads were then added to the sample and incubated for 10 minutes and the cell suspension was made up to a total volume of 2.5mL with HBSS. To obtain uniform suspension, cells were gently mixed and then tubes were placed in our EasySep Magnet for a period of 5 minutes. Any supernatant was discarded and the addition of magnetic beads steps were repeated 3X to obtain a 97% purity level. Following this process protein determination for Western blot analysis immediately commenced.

Western blot

Tissue punches collected from SNc were used to detect changes in proteins primarily related to microglia activation including WAVE2 and CX3CR1, as well as for the dopamine transporter (DAT) and gp91 (marker of oxidative stress), and LRRK2. In the microglia isolation component of this study, only WAVE2 and CX3CR1 western blots were run due to low protein levels. Western blot and protein determination procedures for the SNc (whole and isolated microglia), whole cell lysates were carried in a procedure identical to studies previously described (i.e. from tissue homogenization – protein PVDF membrane transfer) [284].

For WAVE2 assessment, following protein transfer, membranes were placed in tris buffered saline with tween (TBS-T;10 mM Tris-base (pH 8.0), 150 mM sodium chloride, 0.5% Tween-20) overnight. The following day membranes were incubated with a rabbit anti-WAVE2 (1:4000; Cell Signaling) antibody diluted in TBS-T containing 0.5% non-fat dry milk for 1.5 hours. Membranes were then washed using TBS-T a total of three times five minutes each to

remove any unbound primary antibody and then incubated in HRP (horseradish peroxidase) anti-rabbit (1:5000) secondary antibody diluted in TBS-T containing 0.5% non-fat dry milk. After removal of unbound secondary antibody (3 X 5 minutes in TBS-T) protein was then visualized using a chemiluminescent substrate (Western Lightning Plus; Perkin Elmer, Waltham, MA, cat#.NEL102001EA) and then exposed for 5 minutes on a Kodak X-OMAT film. Protein bands were then quantified by densitometry using ImageJ software and desired protein band length was normalized to actin (1:20,000). All incubations occurred at room temperature with gentle shaking.

For DAT, LRRK2, gp91, and CX3CR1 membranes, to determine total protein, after a brief methanol rinse (5 seconds), membranes were incubated in REVERT total protein solution for a period of 5 minutes followed by placement into a REVERT wash solution (6.7% Glacial Acetic Acid, 30% Methanol, in water) two times 2 minutes each. Membranes were then quickly rinsed with distilled water and imaged on our LI-COR Odyssey imaging system on the 700 channel for an exposure period of 2 minutes. Membranes were then rinsed immediately post imaging in tris buffered saline (TBS (pH 7.5 (2 X 5min.)) followed by being blocked with 0.5% fish gelatin (Sigma) in TBS for 60 minutes. Membrane incubation with a rat anti-DAT primary antibody (1:800; Santa Cruz), rabbit anti-CX3CR1(1:1000; Sigma), or rabbit anti-gp91 (1:5000; Abcam), or anti-rat LRRK2 (1: 500; Abcam) for a period of 60 minutes in 0.05% fish gelatin in TBS with 0.1% tween then commenced. Any unbound antibody was removed using 15 mL of TBS-T/membrane at room temperature four times five minutes each. Membranes were then incubated for one hour in infrared conjugate directed against the species the primary

antibody was raised in (rabbit or rat 800, LI-COR) at a concentration of 1:20 000 in 0.5% fish gelatin solution containing 0.2% tween and 0.01% SDS. Membranes were then washed in TBS-T (4 X 5 minutes) followed by 2 X 5 minutes washes in TBS and read on our Licor Odyssy system at the appropriate wavelength for 6 minutes.

Immunostaining

To obtain a representative picture of the brains inflammatory environment 2 days following LPS exposure, tissue was collected in transcardially perfused mice. In particular, it was of interest to us to assess whether or not global LRRK2 ablation protects against microglia activation (as measured by CD68 activity).

Brains were sectioned (40 um thick) on our cryostat and sections were placed in Watson's cryoprotectant (7) and stored at -20 ^{o}C. Upon tissue processing, SNc slices were washed in phosphate buffered saline (PBS; pH 7.2) three times for a period of 5 minutes each, followed by a 1-hour incubation in blocking solution containing 5% normal goat serum (NGS) (5% NGS, 0.4% Triton X, 94.7%PBS); pH 7.2). Following removal of the blocker, slices were then incubated with primary antibodies (anti-mouse TH (1:2000), anti-rat CD68 (1:1000) in a solution containing (5% NGS, 0.3% Triton X, 0.3% BSA, 94.4% PBS) for a period of 2 hours. Sections were then washed in PBS three times for a period of 5 minutes each and then reacted with a secondary Alexa Flour antibody for the appropriate species (mouse Alexa 350 (1:500); rabbit Alex 488 (1:1000)) in a solution containing (5% NGS, 0.3% Triton X, 0.3% BSA, 94.4% PBS). All steps occurred at room temperature.

Microscope Analysis

The signal was visualized with immunofluorescence confocal microscopy using Zeiss image acquisition software (Zeiss LSM 510). All slices were the same distance from bregma. Estimates of microglia activation were unbiasedly obtained via a Zeiss Axioskope 2 Mot Plus (Carl Zeiss, Thornwood, NY, USA) attached to a motorized stage using StereoInvestigator Software on a connected computer (MicroBrightfield, Colchester, VT, USA). Contours of the SNc were drawn based on TH staining. State of microglia cell activation of CD68 sections in the SNc (−3.08, -3.16, -3.28, -3.40, and −3.52) were rated using a scale describe previously, and explained above [168].

Data Analysis

All data in study 2 were analyzed by a 2 (genotype; WT vs. KO) X 2 (injection; saline vs. LPS) ANOVAs followed by Fisher's planned comparisons ($p < 0.05$) where appropriate. All data was analyzed using the statistical software StatView (version 6.0) and differences were considered statistically significant when $p < 0.05$.

Results

Study 1: LPS priming with later paraquat exposure

LRRK2 KO protects against home cage locomotor activity deficits following LPS and paraquat exposure

The LRRK2 KO and WT littermates were infused with LPS (2 μg) or vehicle into the SNc and two days later received the paraquat (10 mg, twice a week for three weeks) or saline injections. It was clear that the animals did not differ in home cage motor activity at baseline prior to receiving the experimental injections (Figure 3 panel A). However, there was a significant overall Genotype X Treatment X Time interaction for home cage activity following the final paraquat injection ($F(1,70) = 4.05$, $p < 0.05$). As shown in Figure 3 panel A, WT mice that were treated with six injections of paraquat had reduced motor scores relative to their paraquat injected LRRK2 KO counterparts, as well as relative to saline treated controls ($p < 0.05$). Moreover, WT mice that were LPS primed and then received paraquat had home cage activity levels reduced further still than those that only had paraquat treatment and again, LRRK2 KO totally prevented this effect ($p < 0.05$).

LPS and paraquat treatments provoked coordinated behavioral impairment in WT but not KO mice

The Rotorod measure of coordinated activity showed a significant interaction between Genotype X Injection ($F(1, 71) = 10.52$, $p < 0.05$). Indeed, the retention time on the rotating drum was significantly reduced in WT mice that received paraquat (irrespective of LPS priming), relative to their paraquat injected LRRK2 counterparts or in relation to saline control animals ($p < 0.05$; Figure 3 panel B).

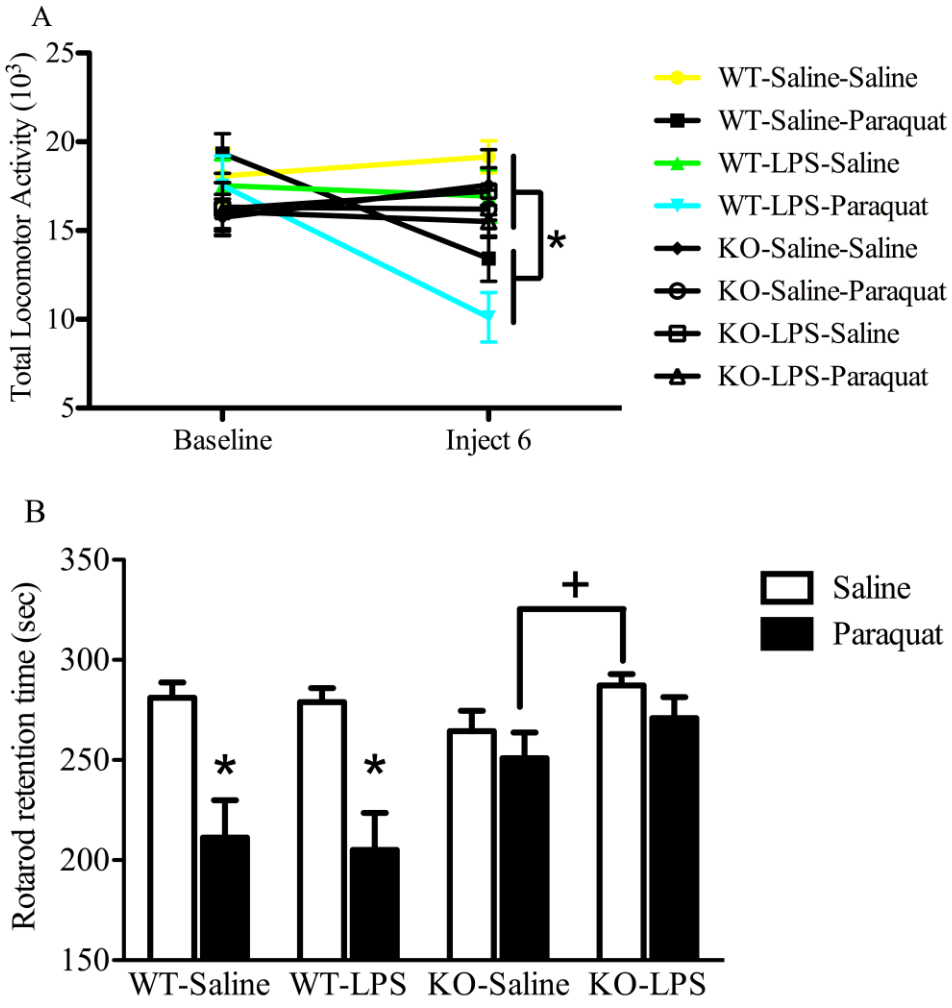

Figure 2: LRRK2 KO prevented toxin induced motor impairment

Impact of LRRK2 on motor functioning. Mice were primed by infusion of LPS (2 ug) or vehicle into the SNc followed two days later by paraquat (10 mg/kg; ip) or saline injections twice/week over three weeks. As shown in panel A, following the final injection, wild type (WT) but not LRRK2 knockout (KO) mice that received LPS + paraquat displayed a significant reduction in home cage activity, relative to the remaining groups. Paraquat alone provoked a significant but more modest motor activity reduction. Panel B shows rotarod retention time, which is a measure of motor coordination. Both paraquat alone and LPS priming followed by paraquat exposure reduced retention time, indicating coordination impairment. However, LRRK2 deficiency totally prevents these deficits. * $p < 0.05$, relative to all other groups.

LPS-paraquat provoked SNc dopamine cell loss is ablated in LRRK2 KO mice

Four days following the last injection mice were sacrificed and while no changes to striatal fiber density were shown in any of our groups (data not shown), a significant Genotype X Injection interaction was evident with regards to the number of surviving TH+ dopamine neurons within the SNc ($F(1, 34) = 7.21$, $p < 0.05$). Specifically, in WT mice, paraquat alone reduced the number of SNc dopamine neurons ($p < 0.05$, relative to saline) and this effect was further enhanced in mice that were primed with LPS and then received paraquat ($p < 0.05$; relative to paraquat alone; Figure 4 panels A-D). Most importantly, neither the LPS + paraquat nor the paraquat alone treatments affected the number of SNc dopamine neurons in the LRRK2 KO mice. Indeed, the LRRK2 LPS and paraquat treated mice did not differ from saline treated controls.

LPS-paraquat provoked SNc glial changes that accompany neurodegeneration

With regards to SNc microglial reactivity state (morphological), the ANOVA revealed a Genotype X Injection interaction ($F(1, 32) = 6.45$, $p < 0.05$). The inset (Figure 4 panel F) shows microglial appearance. The follow up comparisons confirmed that SNc microglia ratings were significantly increased by both the paraquat alone and LPS + paraquat treatment regimens ($p < 0.05$, relative to saline). Interestingly however, LRRK2 KO prevented the impact of LPS + paraquat ($p < 0.05$), but did not prevent the impact of paraquat alone (Figure 4 bottom panel). Thus, LRRK2's role in microglial activation (or at least morphological state) seem to be more aligned with LPS + Paraquat.

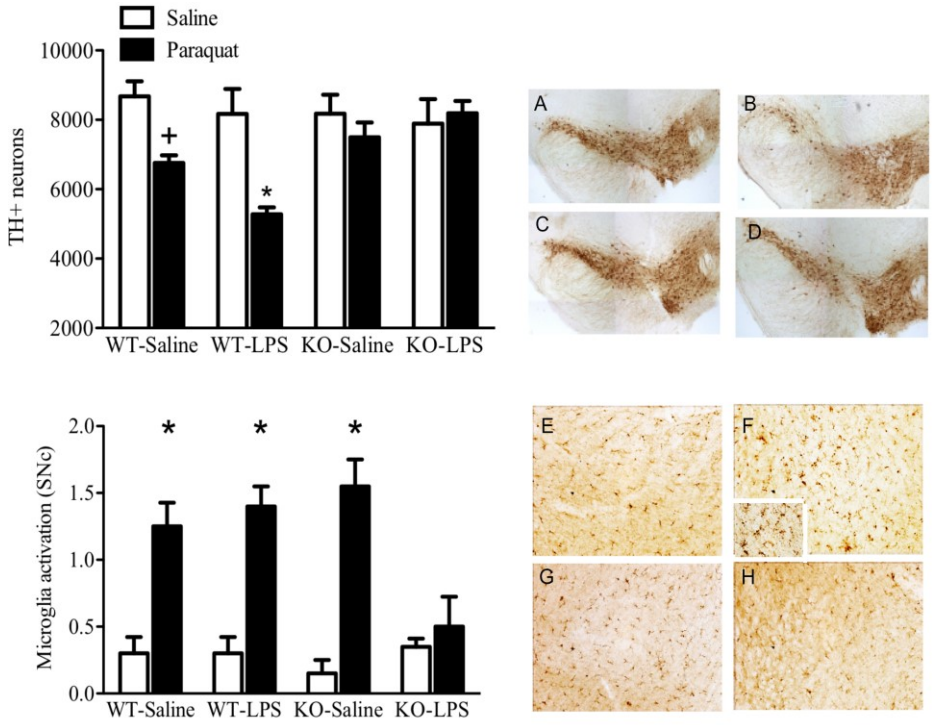

Figure 3: LRRK2 KO was neuroprotective

Neuroprotective effects of LRRK2 ablation. The top left panel displays SNc TH+ dopamine neuron quantification for all treatment groups. Paraquat alone reduced the number of stereologically counted TH+ neurons in wild type (WT) mice, and this effect was further enhanced in the LPS primed WT mice. So, LPS primed mice that received the paraquat regimen two days later had TH+ neuronal counts less than any of the other groups. However, these effects were totally absent in the LRRK2 KO mice. The representative photomicrographs on the right, display TH+ staining for WT mice that received saline (A) or LPS + paraquat (B), as well as LRRK2 knockouts (KO) that likewise received saline (C) or LPS + paraquat (D). The bottom left panel shows quantification of CD68+ microglial ratings for all treatment groups. Both paraquat alone and LPS + paraquat induced a modest increase in microglial morphological state, above saline treatment. Interestingly, LRRK2 KO prevented this effect in the LPS + paraquat mice, but not in those that received paraquat alone. Again, representative photomicrographs show WT mice that received saline (E) of LPS + paraquat (F), as well as KO mice that were saline (G) or LPS + paraquat (H) treated. *$p < 0.05$, relative to saline, +$p < 0.05$, relative to WT LPS + paraquat.

Study 2: LRRK2-related mechanisms of LPS priming of SNc microglia

Microglia activated morphology is altered in LRRK2 KO mice

In order to determine the inflammatory state of microglia following LPS priming (prior to dopamine neuron loss and at the time when our normal paraquat injection regimen begins) we sacrificed mice two days following intra-SNc infusion of either LPS (2 μg) or vehicle. Microglial ratings of morphological state were significantly different between groups ($F(1,12) = 67.13$, $p < 0.001$). Clearly, LPS increased microglial reactive morphology in WT mice but this was totally ablated in the LRKK2 KOs ($p < 0.05$; Figure 5 panels A and B). Importantly, microglial reactivity was much more pronounced at this two-day time point following LPS, as compared to the that of Study 1, wherein mice were sacrificed at a much later time.

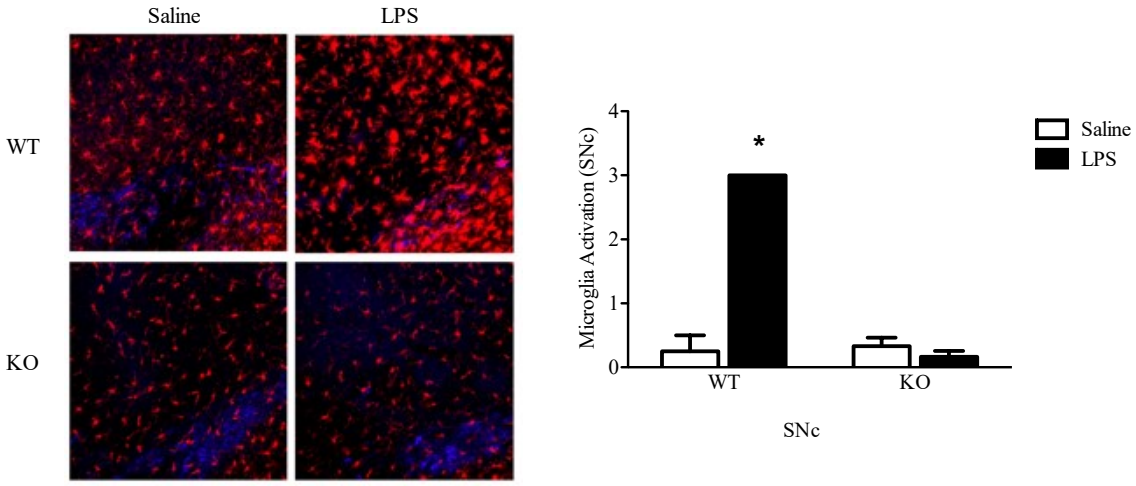

Figure 4: LRRK2 modulated microglia active morphology

Microglia (CD68) immunofluorescence is depicted in the top photomicrograph (A) and quantification in the bottom panel (B). Within the SNc, CD68+ cells from saline (Sal) treated wild type (WT) and LRRK2 null (KO) mice displayed typical ramified fine cell processes that are characteristic of healthy microglia in their normal surveying state. However, LPS markedly changed the morphological state of these cells in the WT but not LRRK2 null mice. Specifically, the endotoxin promoted a state, such that CD68+ cells of WT mice had thicker soma, retracted fiber projections and a bushy appearance. The most activated cells were amoeboid-like in appearance and reflect either the most "activated" microglial state or alternatively could be invading macrophages/moncytes. Yet, most CD68+ cells within the SNc had a more intermediate appearance, characteristic of microglia in various states of activation. The bottom panel show the quantification using our well validated rating scale. Clearly, LPS selectively induced augmented morphological ratings of microglial activation in WT mice, relative to the remaining three groups. *$p < 0.05$, relative to saline treatment.

LRRK2 KO further alters microglia phenotype basally and in endotoxin exposed mice

To further assess the microglia cell phenotype, microglia were extracted from the SNc using a magnetic bead strategy (described in methods). These microglia displayed a significant Genotype X Injection interaction for WAVE2 levels ($F(1,12) = 4.543$, $p < 0.05$). Specifically, as shown in Figure 6 panel A, WT mice showed a marked increased in SNc WAVE2 protein levels at two days following the LPS infusion ($p < 0.05$). However, LRRK2 null mice showed a dramatically blunted response compared to WT animals ($p < 0.05$). Furthermore, CX3CR1 SNc levels within isolated microglia were also significantly altered by Genotype ($F(1,12) = 50.13$, $p < 0.05$). However, in contrast to WAVE2, SNc CX3CR1 levels were not affected by LPS treatment, but they were markedly elevated in LRRK2 null mice compared to WT animals (Figure 6 panel B). Finally, we sought to confirm that LPS actually induced the LRRK2 protein within the SNc, given that basal levels within this brain region are exceedingly difficult to detect. To this end, LPS did indeed significantly increase SNc LRRK2 protein and as expected, this effect was totally absent in LRRK2 null mice ($F(1,12) = 41.39$, $p < 0.05$) (Figure 6 panel C).

In addition to microglial responses, we assessed how LPS priming might affect dopaminergic neurons. To this end, we found a significant Genotype X Injection interaction for SNc levels of the dopamine transporter, DAT ($F(1,19) = 23.116$, $p < 0.05$). Interestingly, while LPS reduced DAT levels in WT mice, it actually increased levels in the LRRK2 null animals ($p < 0.05$; Figure 6 panel D). Thus, LRRK2 dramatically influences the impact of LPS primed

microglia on DAT neuronal availability. Assessment of the gp91 catalytic subunit of the NADPH oxidase microglial enzyme was also assessed however this revealed no differences between the groups (data not shown).

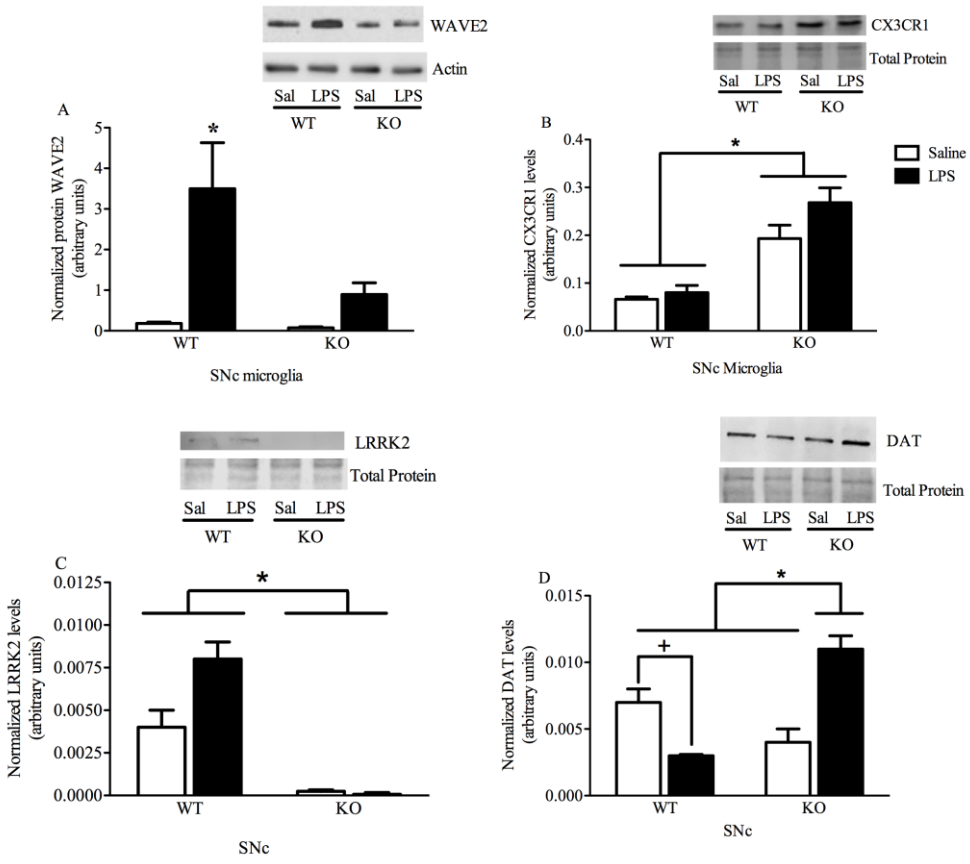

Figure 5: LRRK2 modulated microglia phenotype

Microglia were isolated (A-B; using magnetic beads; see methods) from mice that were primed with LPS (2 ug) or vehicle by infusion into the SNc two days earlier. The LPS treatment markedly increased SNc WAVE2 levels in WT mice, but this effect was prevented by LRRK2 KO (A) (* $p < 0.05$ relative to all other groups). In contrast, LRRK2 KO increased microglia CX3CR1 levels in the SNc in the absence of an LPS effect (B) (*$p < 0.05$ relative to WT mice irrespective of genotype). Levels of the LRRK2 protein itself were observed in WT but not KO mice ($p < 0.05$ relative to KO mice irrespective of treatment). However it is important to note that these levels were almost undetectable in untreated WT mice, but seemed to have the most elevation when LPS was infused two days earlier (C). DAT levels in whole SNc tissue (D), were reduced by LPS in WT mice, but were significantly increased in LRRK2 KO animals treated with the endotoxin. (* $p < 0.05$, relative to saline treatment.)

Discussion

Our experiments revealed the importance of LRRK2 in PD models involving LPS and paraquat exposure. Indeed, it has been our contention that a variety of different categories of stressors, from psychological to chemical to immune, come to collectively influence the development of PD, as well as its clinical course and type of co-morbidity [283], [303], [351]. Hence, the immune and chemical stressors, LPS and paraquat (respectively), may act as triggers eliciting a wide range of PD relevant pathology through parallel inflammatory and oxidative pathways. We presently found that LRRK2 deficient mice were protected against the loss of SNc dopamine neurons induced by paraquat alone, as well as LPS priming followed by paraquat exposure. Moreover, this effect was reflected in preservation of motor functioning, suggesting that LRRK2 plays an important role in shaping the impact of environmental insults upon PD-like processes.

The neuroprotective effects of LRRK2 knockout might be mediated by a blunting of local microglial activity, or possibly through inhibition of peripheral immune cell infiltration into the SNc. Indeed, LRRK2 deficiency diminished the morphological characteristics reflective of a microglial activation state that were evident in wild type mice primed with LPS. Similarly, SNc CD11b+ isolated cells from LRRK2 null mice displayed an altered expression of WAVE2 and CX3CR1 immune regulatory proteins. Hence, it appears that LRRK2 modulates SNc microglia reactivity to direct LPS infusion.

We presently found that microglia isolated from mice that received intra-SNc LPS priming two days prior to receiving paraquat (i.e. long before dopamine neuron loss and at a time when paraquat administration would just begin) showed an inflammatory phenotype in wild type mice that was absent in LRRK2 knockouts. Indeed, microglia from LPS treated wild type mice displayed morphologically activated appearance, whereas this response was markedly blunted in LRRK2 null mice. These morphological changes were likely mediated by the WAVE2 pathway, as it was greatly elevated in microglia isolated from LPS primed wild type animals, but diminished by LRRK2 ablation. Indeed, WAVE2 is a critical mediator of pro-inflammatory actions that require a morphological change in microglial cytoskeleton. It is a Rac1 effector molecule that promotes actin polymerization and consequent reorganization of the cytoskeleton [347], [352]. Such remodeling is required for phagocytosis and motility, as well as possibly the release of inflammatory secreteagogues [343], [348]. Further, members of our group have recently shown that WAVE2 was crucially involved in the phagocytic response of bone marrow derived macrophages (BMDMs) and was important for LRRK2 mediated pathology in mice bearing the G2019S mutation [270].

The recent findings from our group were the first showing that the WAVE2 pathway is regulated by LRRK2 and is important for inflammatory processes linked to PD [270]. The present study extends these findings and is the first to implicate LRRK2-WAVE2 in an *in vivo* double-hit model of PD. We also extend the findings that LRRK2 knockout basally increased levels of the fractalkine receptor, CX3CR1, which normally has important anti-inflammatory consequences, and in this case may be important in conferring protection in our models. In

regard to the WAVE2 pathway, our findings promote the possibility that LRRK2-WAVE2 signaling could be a primary mediator of microglial or even inflammatory phagocyte phenotype. Also of critical importance is the fact that WAVE2 mediates the endocytosis and subsequent cellular uptake of the bacterial pathogens [353], [354], and hence, might have been critical in modulating a similar response on SNc microglia exposed to LPS. Indeed, besides the well known membrane induced TLR4-MyD88-NFkB pathway, the internalization of LPS secondarily elicits CD14-TRIF-interferon signaling [355]. We also can not rule out other mechanisms as possible low levels of contaminant in the LPS could conceivably impact non-TLR4 processes. Nevertheless, the blunted WAVE2 response observed in LRRK2 knockouts may have reduced the internalization of LPS and hence, limited the extent of its general inflammatory signaling.

In concert with the WAVE2 changes, microglia isolated from the LRRK2 knockout mice had greatly augmented CX3CR1 levels. However, unlike WAVE2, this elevation was basally apparent and not significantly altered by LPS. This chemokine receptor is strongly implicated in microglial anti-inflammatory responses induced by its chemokine ligand, fractalkine [285], [345], [356], [357]. Hence, LRRK2 deletion could promote a basal shift in microglial ability to coordinate anti-inflammatory responses. In fact, LRRK2 may impart some of its pro-inflammatory actions by down-regulating CX3CR1 and the associated NFAT transcription factor [198], [358], [359]. Whatever the case, our data suggest that LRRK2 is likely involved in mediating microglial reactivity through CX3CR1 and WAVE2, which can impact dopamine neuronal survival in the face of subsequent challenge (e.g. paraquat).

Of course, it should also be underscored that paraquat alone produces SNc neuronal loss and functional deficits (albeit usually less than LPS + paraquat). So the fact that LRRK2 deficiency was protective in this case indicates that LRRK2 also has the ability to modulate pro-death mechanisms independent or additional to any of the LPS induced central inflammatory pathways. This raises the possibility that LRRK2 also modulates processes intrinsic to the dopamine neurons. Evidence for this is the fact that recent reports indicate that the G2019S LRRK2 mutation altered dopaminergic neurotransmission [234], [360], [361].

The exact downstream of inflammatory mechanisms that could kill dopamine neurons are not entirely clear but would presumably be the result of the release of soluble factors and/or direct glial-neuron interactions. While the number of intracellular neuronal pro-death pathways that could be activated is substantial, alterations in the dopamine metabolic machinery would be high on the list of suspects. Along these lines, we found that dopamine transporter (DAT) levels on SNc dopamine neurons were greatly reduced by LPS priming, but conversely, were enhanced in LRRK2 knockouts exposed to the endotoxin. Such changes in DAT levels following LPS priming could have profound consequences on dopamine neuron survival in response to subsequent paraquat exposure. In fact, epidemiological studies reported that certain DAT polymorphisms together with pesticide exposure greatly increased the likelihood of PD, compared to the risk imparted by either of these factors alone [70], [362]. Furthermore, the DAT can influence paraquat uptake and oxidative potential in dopamine neurons [51].

In effect, variations in DAT levels together with dopamine turnover could influence the production of metabolic reactive oxidative species (possibly adding to those already released from activated microglia). This would be consistent with reports that LRRK2 is known to modulate lysosomal functioning and may influence SNc dopamine neuronal survival through autosomal protein degradation pathways [363], [364]. So, essentially LPS immune priming might impact the ability of subsequently applied paraquat to: (1) enter the dopamine neurons, (2) modulate redox state and/or (3) affect intracellular "detoxification apparatus".

Conclusions: We found that LPS priming sensitized the microglia phenotype and increased dopaminergic degeneration to subsequent paraquat exposure. LRRK2 knockout completely prevented this effect, and modulated WAVE2 and CX3CR1 signaling in isolated microglia. While we presently show that wild type LRRK2 is crucial for PD-like neuropathology and inflammatory tone, we also must acknowledge that substantial attention is being devoted to the potential deleterious consequences of specific LRRK2 mutations [242], [365], [366]. In particular, the most common LRRK2 mutation, G2019S, was associated with a diverse phenotype and it remains to be determined whether the mutation differentially modulates LPS and paraquat processes currently reported. At the very least, the present work highlights the potential for endogenous LRRK2 to participate in multiple levels of disease initiation relevant for PD and its comorbidities. More specifically, LRRK2 might act through WAVE2 to regulate microglial phenotype in the face of inflammatory challenge and hence, influence the vulnerability of dopamine neurons to further insults.

Chapter 3: Age-dependent paraquat toxicity: Effects of LRRK2 KO

Preface

This final study of this dissertation was conducted in order to determine if LRRK2 KO influences the overall health and general level of toxicity typically ascribed to paraquat. Moreover, in this study we used older mice (~8 months) than our previous studies (3 months), to determine whether any of LRRK's effects are age-dependent, and to ascertain whether this factor might also impact the toxic effects of paraquat exposure (not only with regards to the brain, but also at peripheral sites). Indeed, as will be outlined, in our preliminary studies using mice ~8 months of age, we did notice that a 9 injection paraquat protocol caused a significant amount of sickness and mortality in a large portion of such older male mice, and we also collected some preliminary data showing that LRRK2 KO prevented this paraquat induced toxicity. Owing to the enhanced mortality with our 9 injection paraquat procedure in mice of this age, we presently opted for 6 paraquat injections in order to capture this effect. Indeed, the mortality/sickness was typically observed by the 5^{th} injection.

Highlights

- LRRK2 ablation prevented paraquat induced motor and non-motor behavioral changes in aged mice.
- LRRK2 was important for the generally “toxic” overall profile induced by paraquat.

Abstract

Parkinson's disease (PD) is a chronic progressive neurodegenerative disorder characterized by the presence of motor and non-motor behavioural deficits. Much attention has been afforded to the commonly used herbicide, paraquat, as an environmental risk factor for the development of PD. Paraquat exposure in rodents recapitulates many of the hallmark pathological features of the disease. Additionally, neuroinflammatory and oxidative processes are critically important for paraquat-induced brain and behavioural changes. The leucine rich repeat kinase 2 (LRRK2) gene, of which mutations are seen in both inherited and sporadic PD cases, has been shown to modulate neuroinflammatory processes to different insults, and we have previously shown that global ablation of the protein results in nigrostriatal protection against paraquat, perhaps due to LRRK2 mediated control of active central inflammatory response processes normally provoked against the toxin. Accordingly, in the current study it was of interest to us to further investigate whether or not LRRK2 ablation protects against paraquat induced toxicity in mice typically older than what we use in our models, who are more vulnerable to toxicant exposure due to the age related alterations imparted on biological systems. To this end, we found that paraquat clearly provoked a marked sickness profile in mice, characterized by reduced motor activity and impaired species-typical behaviours and these effects were accompanied by signs of inflammation and organ pathology. Importantly, LRRK2 ablation prevented not only the sickness and behavioral consequences of paraquat in the aged mice, but also organ pathology,

stress hormone and inflammatory markers. We are the first to show the importance of LRRK2 in such a multi-hit model of PD that involves toxin exposure in the context of aging.

Introduction

It has been supported that environmental exposure to the herbicide paraquat increases the risk of Parkinson's disease (PD) [286], [367]. It is likely that exposure to the PD related toxin in humans, generally occurs through a combination of many routes including via subcutaneous absorption, or orally through inhalation [368]. In rodents, with controlled administration of paraquat via intraperitoneal injection, the toxicant infiltrates the brain and accumulates over relatively long periods of time [295], [369]. Following paraquat exposure, rodents display a loss of nigrostriatal dopamine neurons and signs of excessive microglial activation [51], [60], [295]. The paraquat induced dopaminergic cell loss in the substantia nigra (SNc) appears to be dose-dependent, and typically occurs with doses that don't elicit toxic effects on peripheral tissues depending on age of exposure [370]. This is important since high doses of paraquat can directly damage organs, most notably the liver and lungs often causing death [370], [371]. Our own lab for example, has demonstrated that six or nine 10 mg/kg injections of paraquat in mice is able to induce an ~25% loss of dopamine neurons in the midbrain [45], [46], [168]. In fact, as little as 3 injections can induce cell loss in mice depending on their genetic background [313], [319], [370]. Additionally, we have shown that pretreated mice with the endotoxin lipopolysaccharide (LPS), increases the impact of subsequent paraquat exposure resulting in greater than 40% loss of SNc dopamine neurons [46].

Since aging is the number one unequivocal risk factor for PD provocation [372], [373], it is important to evaluate the impact of toxicants, like paraquat, in aged animals. Indeed, it is well

known that as one ages, the body undergoes “wear and tear” that often result in organ or system specific vulnerabilities [284]. For example, aging can induce alterations in blood brain barrier (BBB) permeability, hypothalamic-pituitary-adrenal (HPA) axis activity, microglia activation, and widespread reductions in the neuroplastic factor brain-derived neurotrophic factor (BDNF) [115], [374]–[376]. In the context of PD, these age induced “wear and tear” alterations may modulate nigrostriatal vulnerability to environmental insults such as paraquat [377], [378]. Indeed, a number of studies have demonstrated the enhanced neurodegenerative effects of the toxin in aged mice (i.e. 8 months vs. 3 months) [370], [379]. This is in line with the multi-hit hypothesis of the disease which suggests that PD development likely involves the collective contribution of a spectrum of stressors (including psychological, immune, or chemical) overtime, which in some cases interact with aging induced alterations and genetic factors/vulnerabilities to result in a PD diagnosis [23].

Mutations in LRRK2 have been implicated as a genetic risk factor for PD [3]. However, given the incomplete penetrance of these mutations in the disease, coupled with the low concordance in relatives, the notion that genetic mutations likely interact with environmental triggers is strongly supported [31]. While it has been known for over a decade that LRRK2 mutations represent a causal factor in the disease, the “normal” role of LRRK2 is not yet fully understood. However, a number of studies have implicated LRRK2 in the regulation of a vast array of intracellular factors important for homeostatic functions, including cytoskeletal dynamic [252], vesicular trafficking [253], and apoptosis [254].

Growing evidence suggests that the most prominent role of LRRK2 is in the functioning of various aspects of inflammatory immunity [3]. In particular, some reports suggest that the highest levels of LRRK2 protein are found in a variety of immune cells including B cells [380], T cells [380], CD16+ monocytes [380], as well as microglia [257]. Moreover, as just described in the previous chapter of this thesis, we found that LRRK2 deficiency protected mice against the inflammatory and neurodegenerative impact of LPS and paraquat. Accordingly, in our current study, it was of interest to further investigate whether or not LRRK2 ablation protects against paraquat induced toxicity in older mice that might be more vulnerable to the overall toxicity of the herbicide. Given that paraquat has also been shown to influence processes outside of the nigrostriatal system, and in particular HPA activity, glucocorticoid receptor and neurotrophic factor (i.e. BDNF) hippocampal expression which might hold important implications for the co-morbid aspects of the disease, it was also of interest to us to evaluate whether or not LRRK2 might be important in the regulation of these processes. Consistent with our hypothesis, LRRK2 ablation did indeed confer protection against the toxic effects of paraquat on behavior, organ pathology, and inflammatory outcomes. This suggests that LRRK2 might be an important mediator of overall inflammatory/oxidative toxicity stemming from oxidative environmental challenge.

Materials and Methods

General Experimental Design

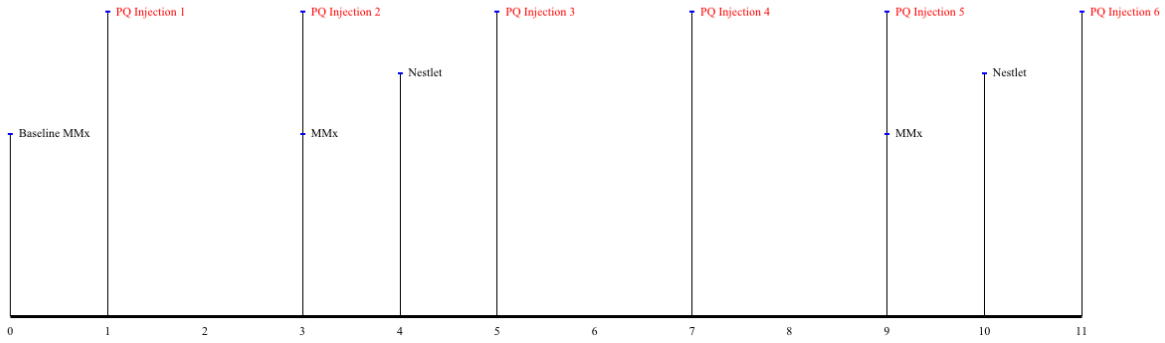

Figure 1: Schematic timeline of study

Schematic timeline of study. (MMx = Micromax; PQ = paraquat)

A schematic timeline for the study can be seen in Figure 1. The current study was undertaken to assess whether LRRK2 might have a role in the toxic effects of paraquat in mice older than what we typically use in our lab. In this study, we were interested in not only evaluating the central effects of the toxin, but also evaluating whether LRRK2 ablation protects against the toxicant induced peripheral inflammatory effects. Importantly, these interests came as a result of a preliminary study conducted which was designed to test whether our findings from a previous study showing that global LRRK2 ablation protects against the motor behavioral and microglia mediated SNc dopamine neuronal cell loss induced by paraquat, or central LPS + paraquat exposure could be replicated in older mice (as age renders one more

susceptible toxicant exposure). Intriguingly, this preliminary study showed that the combination of central LPS exposure in the SNc followed by paraquat exposure two days later (Figure 2), as well as paraquat exposure alone, caused a significant degree of mortality in these older wild type but not LRRK2 null mice. Hence, we focused on the toxicity induced by paraquat alone to assess whether LRRK2 is protective against behavioral, central, and systemic outcomes.

To this end, forty male (n = 8-10/group) LRRK2 knockout (KO) or wild type (WT) littermate controls aged ~8 months were obtained from our in-house breeding colony. All genotyping procedures were conducted according to the methods described in the methodas described previously. At two months of age, mice were individually placed in standard polypropylene cages (27 × 21 × 14 cm) in which they were provided an *ad libitum* diet of Ralston Purina chow (St. Louis, MO) in a temperature controlled room (maintained at ~21°C) that had a 12h light/dark cycle with lights off at 20:00 hours. The Carleton University Committee for Animal Care approved all experimental procedures and complied with the guidelines set out by the Canadian Council for the Use and Care of Animals in Research.

All animals were randomly assigned to one of four experimental conditions (WT/Saline; WT/Paraquat; KO/Saline; KO/Paraquat) and saline (Sigma) or paraquat (10 mg/kg; 1,1′-dimethyl-4,4-bipyridinium dichloride; Sigma Aldrich, St Louis, MO, USA). Injections were given every other day for a total of 6 injections. Animal weights, sickness behaviors, and motor behaviour alterations assessing home cage locomotor activity, and fine movement coordination

(also a measure of motivational processes) were carried out at various time points as described below. At the end of the experimental paradigm, all animals were rapidly decapitated 1 hour following the final injection to measure organ pathology (i.e. organ weights, splenic inflammatory cell infiltration), changes in HPA activity (as measured looking at plasma corticosterone levels and glucocorticoid receptor expression in the hippocampus), trophic factor expression in the SNc, striatum, and hippocampus, and additional inflammatory outcomes in the SNc.

Sickness Behaviour

Sickness symptoms (e.g. ptosis, curled body posture, piloerection) were assessed daily throughout the injection regimen, as previously described [381]. All animals were assessed for the presence of the following symptoms: ptosis (drooping eyelids), piloerection (ruffled and greasy ungroomed fur), curled body posture, and diminished locomotion and/or exploratory behaviour. Upon assessment, a score based on the number of symptoms present (0 = no sickness symptoms, 1= one symptom, 2= two symptoms, 3 = three symptoms) was applied. Ratings were scored by an observer blind to all experimental conditions.

Nestlet test

In order to assess goal-directed behaviour alterations involving fine motor coordination skills in saline or paraquat LRRK2 WT or KO mice, the nestlet test was used, as previously described [357]. One day following the 2nd and 5th injection (Day 4 and 10 respectively), mice

were placed into a new standard polypropylene cages (27 × 21 × 14 cm) containing one fully intact nestlet (Ancare, Bellmore, NY) beginning at 08:30. Mice nestlet building behaviour was then examined based on the quality of nest built at 1, 3, 5 and 24 hours by an investigator blind to all experimental conditions. All scoring was based on the nestlet likert rating scale whereby scores range from 0 (untouched intact nest) – 6 (perfectly developed nest) [357]. Ratings were scored by an observer blind to all experimental conditions.

Home cage locomotor activity

Spontaneous home cage locomotor activity was measured over a complete 12 hour dark cycle using our MMx beam-break apparatus as described from other studies in our lab [291]. Activity in a rodents home cage is determined based on the number of infrared beam-breaks made based on 16 infrared wavelengths that originate external to the home cage. Following removal of the nestlet, all animals acclimated to our behavioural testing room for 30 minutes and measurements of home cage locomotor activity occurred once at baseline (Day 0), then again the evening of the 2nd and 5th injection (Day 3 and 9 respectively).

Brain dissection and tissue extraction

Following rapid decapitation, brains were excised and micro-punches were taken using a chilled microdissecting block containing slots 0.5mm apart. Tissue was immediately frozen upon dissection and stored at -80^{o}C until processing. Additionally, immediately following sacrifice the animals left lung, spleen, left kidney, and liver, were extracted from the cavity and

any excess fat was removed and organs were promptly weighed. The lungs (middle lobe), spleen, and liver were placed on dry ice and stored at -80ºC until processing.

Plasma corticosterone assay

At the time of decapitation, trunk blood from all of the animals, was collected in tubes containing 10 µg ethylenediaminetetraacetic acid (EDTA). Samples were centrifuged (3000g for 8 min) and the plasma removed and stored in aliquots at −80 °C for later corticosterone determination with commercially available radioimmunoassay kits (ICN Biomedicals, CA, USA). Samples were assayed in duplicate within a single run to control for inter-assay variability; the intra-assay variability was less than 10%.

Western blot

The SNc tissue punches collected were used to detect levels BDNF (marker of plasticity), gp91 (marker of oxidative stress), as well as chemokine receptor 1 (CX3CR1; anti-inflammatory associated receptor) and WASP-family verproline homologous protein-2 (WAVE2; marker of "activation" state of phagocytic cells). While it was our intention to detect levels of the aforementioned proteins in the striatum, only levels of BDNF were determined due to unintentional degradation of the samples. Additionally in order to ascertain whether LRRK2 ablation impacts the effects of paraquat on regions outside of the nigrostriatal system (in particular the hippocampus) we assessed levels of BDNF, and glucocorticoid receptor (GR) expression in the area. Moreover, it was also of interest to us to ascertain whether LRRK2

ablation might impact the toxic effects of paraquat exposure on peripheral organs. As such Western Blot analysis was carried out assessing WAVE2 levels in liver and lungs. All western blot and protein determination and membrane transfer procedures for the striatum, SNc, hippocampus, liver, and lung, were carried out in a procedure identical to studies previously described in our lab [284].

For assessment of WAVE2 levels in the liver, lung, and SNc, following protein transfer, and appropriate washes in TBS-T (3 X 5 min each), membranes were incubated in a TBS-T solution containing 0.5% non-fat dry milk with rabbit anti-WAVE2 (1:4000; Cell Signaling) antibody for 1.5 hours. Following primary incubation, membranes were again washed (3X5 min each) in TBS-T and then an HRP anti-rabbit (1:5000; Sigma) secondary antibody (diluted in in TBS-T containing 0.5% non-fat dry milk) was applied. Following washes in TBS-T (3X5 min each) the WAVE2 protein was visualized using a chemiluminescent substrate (Western Lightning Plus; Perkin Elmer, Waltham, MA, cat#.NEL102001EA) and exposed for 5 minutes on a Kodak X-OMAT film. Protein bands were quantified by densitometry using ImageJ software and the protein band was normalized to actin (1:20,000; Sigma). All incubations occurred at room temperature with gentle shaking.

For BDNF, gp91, and CX3CR1 detection in the SNc, BDNF detection in the striatum, and BDNF and GR expression in the hippocampus, total protein was determined using a REVERT total protein solution as previously described [284]. Following determination of total protein levels, and appropriate washes in tris buffered saline (TBS; pH 7.5; 2 X 5 min each), membranes

were blocked for 60 minutes in a TBS solution containing 0.5% fish gelatin (Sigma). Membranes were then placed in a O.5% fish gelatin TBS solution containing either a mouse anti-BDNF primary antibody (1:1000; R & D Systems), rabbit anti-CX3CR1(1:1000; Abcam), or rabbit anti-gp91 (1:5000; Abcam), GR (1:500; Sigma), for a period of 90 minutes in 0.05% fish gelatin in TBS with 0.1% tween. Following incubation in the primary antibody and removal of any unbound antibody, membranes were incubated \in infrared conjugate directed against the species the primary antibody was raised in (mouse or rabbit 800, LI-COR) at a concentration of 1:20 000 in a 0.5% fish gelatin TBS solution containing 0.2% tween and 0.01% SDS. Following removal of any unbound antibody protein bands were read on our Licor Odyssy system at the appropriate wavelength for 6 minutes.

Data Analysis

All data were analyzed by 2 (Genotype; WT vs. KO) X 2 (Injection; saline vs. paraquat) ANOVAs followed by Fisher's planned comparisons ($p < 0.05$) where appropriate. Additionally, total home cage locomotor activity, weights, sickness, and nestlet building behaviour, was completed using appropriate repeated measures ANOVA's conducted with *Time* as the 3rd independent variable followed by the relevant posthoc analysis. While all mice were exposed to baseline measurements for total home cage activity, we were unable to include this data in our analysis due to equipment failure. All data was analyzed using the statistical software StatView (version 6.0) and differences were considered statistically significant when $p < 0.05$.

Results

LRRK2 KO protects against paraquat and LPS + paraquat induced mortality

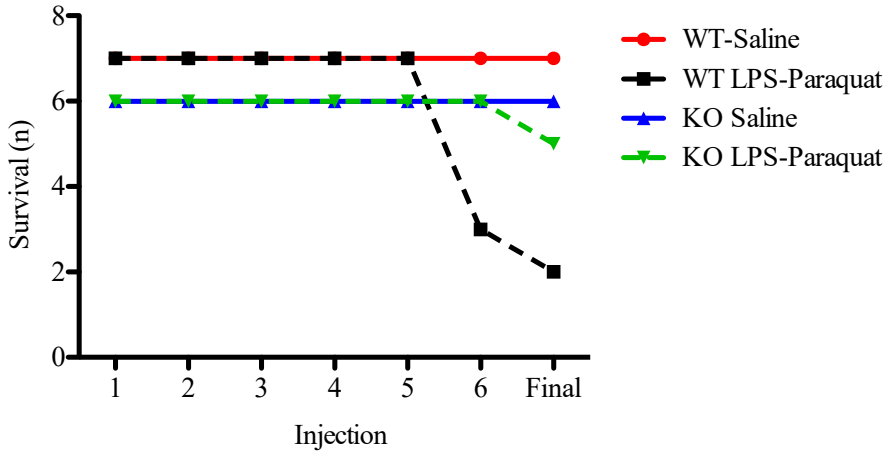

Figure 2: LRRK2 KO protects against toxin induced mortality.

This study was undertaken to assess whether LRRK2 might have a role in the peripheral inflammatory effects of toxicant exposure. Importantly, a preliminary study showed that the combination of LPS + paraquat caused a significant degree of mortality in ~8 month old wild type (WT) but not LRRK2 knockout (KO) mice (Figure 2A). Moreover, not displayed here, paraquat alone also caused a significant degree of mortality that was not observed in LRRK2 ablated animals, suggesting that LRRK2 may play a role in paraquat induced toxicity.

LRRK2 KO protects against paraquat induced weight loss and sickness behavior

The repeated measures two-way ANOVAs revealed a Genotype X Injection X Time interaction for animal weights ($F(5, 160) = 8.319$, $p < 0.001$) and scores of sickness ($F(4,128) = 10.642$, $p < 0.001$) (Figure 3 panel A and B respectively). Follow up post hoc analyses revealed that while all animals maintained body weight over time, beginning one day after the 5th injection, WT mice exposed to paraquat had significantly lower weight and displayed higher sickness scores, relative to their saline exposed littermates ($p < 0.05$), as well as to LRRK2 null mice exposed to the toxin ($p < 0.05$), which continued until our mice were euthanized ($p < 0.05$). These findings are certainly consistent with the protection from mortality observed in LRRK2 knockouts in our preliminary LPS + paraquat study just mentioned.

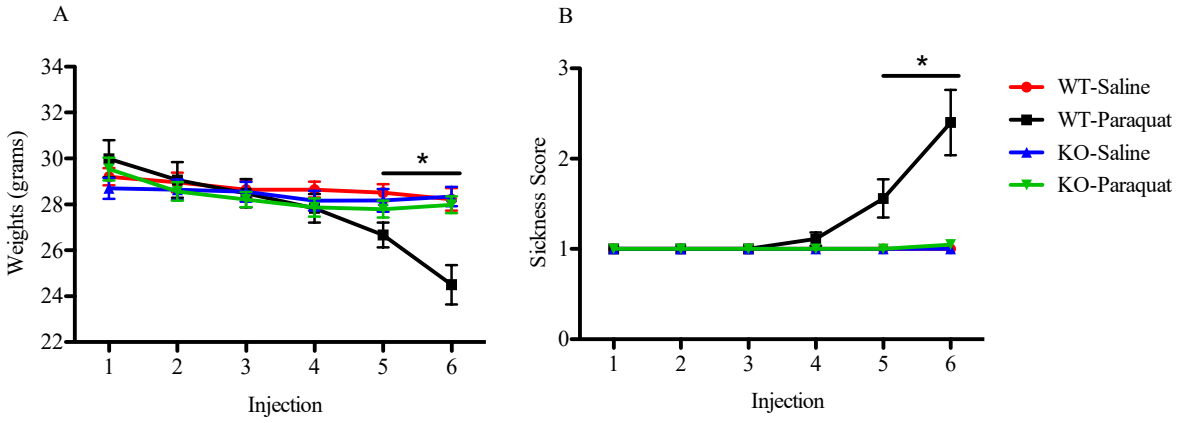

Figure 3: LRRK2 knockout prevented toxin induced weight loss and sickness profile.

Impact of LRRK2 on paraquat induced weight loss and sickness behavior in mice aged 7-8 months. By the 6th paraquat (10 mg/kg; ip) injection in aged mice, marked weight loss (A) and sickness (B) were highly evident in WT mice. However, LRRK2 (KO) deficiency abrogated these effects. Sickness was a calculated as a composite score by blind raters including ptosis, piloerection, lethargy and curled body posture. * $p < 0.05$ all other groups in comparison to WT paraquat treated mice. All data is expressed as mean ± SEM.

Paraquat treatment provoked fine motor and home cage motor behavioral impairment in WT but not KO mice, likely a result of toxicant induced sickness

In regard to total home cage locomotor activity analyzed the evening following the 2nd and 5th injection, results show a significant effect of Time ($F(1,25) = 11.440$, $p < 0.01$), such that mice had lower home cage activity scores as time increased (Figure 4 panel A). Additionally, consistent with weight and sickness scores, a Genotype X Injection interaction was observed ($F(3,25) = 3.196$, $p < 0.05$), such that WT but not KO mice exposed to paraquat had a reduction in total home cage activity relative to all other groups as revealed with post hoc comparisons ($p < 0.05$).

We also assessed nestlet (essentially the animal's in cage material that it typically uses to construct its nest) building behavior, as this requires fine motor skills and can also be taken as an index of stress, or motivational processes. Overall results of the repeated measures two-way ANOVA demonstrated no significant effects following the 2nd injection apart from time (data not shown). However, a significant effect of Time in regards to nestlet building behavior was observed after the fifth injection ($F(3,93) = 56.598$, $p < 0.001$). The repeated measures two-way ANOVA also showed a Genotype X Injection interaction ($F(3,31) = 4.846$) and follow up comparisons revealed WT paraquat treated mice had significantly lower nestlet building scores relative to all other groups ($p < 0.05$; Figure 4 panel B).

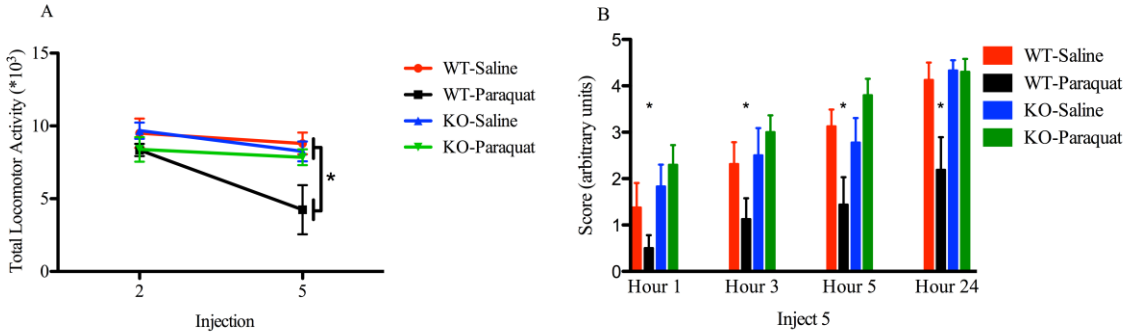

Figure 4: LRRK2 knockout prevented toxin induced nestlet building and home cage locomotor impairment.

Impact of LRRK2 on motor functioning induced by our paraquat injection regimen. In parallel with our sickness behaviours, home cage motor activity was likewise reduced in the aged paraquat treated wild type (WT) mice but not in the LRRK2 knockout (KO) animals (panel A). Paraquat also reduced interactions with their nestlet and the complexity of the nest produced (a measure of fine motor skills) in WT but not LRRK2 null mice (panel B). * $p < 0.05$ relative to all other groups. All data is expressed as mean ± SEM.

LRRK2 KO blunts paraquat induced increase of plasma corticosterone levels

Plasma corticosterone was assessed as an index of stress state of mice and is also a useful measure that usually correlates with inflammatory sickness profiles. Once again, a significant Genotype X Injection interaction was evident ($F(1,32) = 3.883$, $p = 0.05$). Paralleling the sickness syndrome, and nestlet building scores, Figure 5 (panel A) shows that paraquat clearly had a stressor-like effect as reflected by markedly elevated plasma corticosterone levels in WT mice, relative to saline injection ($p < 0.05$). However, this effect was dramatically blunted in the LRRK2 KOs ($p < 0.05$). Our data also indicated that basally the LRRK2 KO saline treated mice had significantly lower levels than WT saline and KO paraquat treated mice ($p < 0.05$).

LRRK2 KO does not alter paraquat induced reduction of hippocampal BDNF levels

A significant effect of Injection was observed in regard to hippocampal BDNF levels ($F(1,20) = 6.831$, $p < 0.05$) such that paraquat exposure caused a reduction in trophic factor expression. No main effect of Genotype or Genotype X Injection interaction was observed. Accordingly LRRK2 ablation did not alter paraquats effects on hippocampal expression of BDNF (Figure 5 panel B).

LRRK2 KO does not alter paraquat provoked reduction in hippocampal GR levels. LRRK2 null animals display lower levels of the receptor

A significant interaction was observed in regard to total GR levels ($F(1,16) = 5.631$, $p < 0.05$). As shown in Figure 5 (panel C), saline treated WT mice had higher levels of GR

relative to all other groups ($p < 0.05$). As such, it appears that paraquat exposure caused a reduction in the protein, and while LRRK2 ablation did not alter this effect, LRRK2 null animals had lower levels of GR relative to their WT littermates ($p < 0.05$).

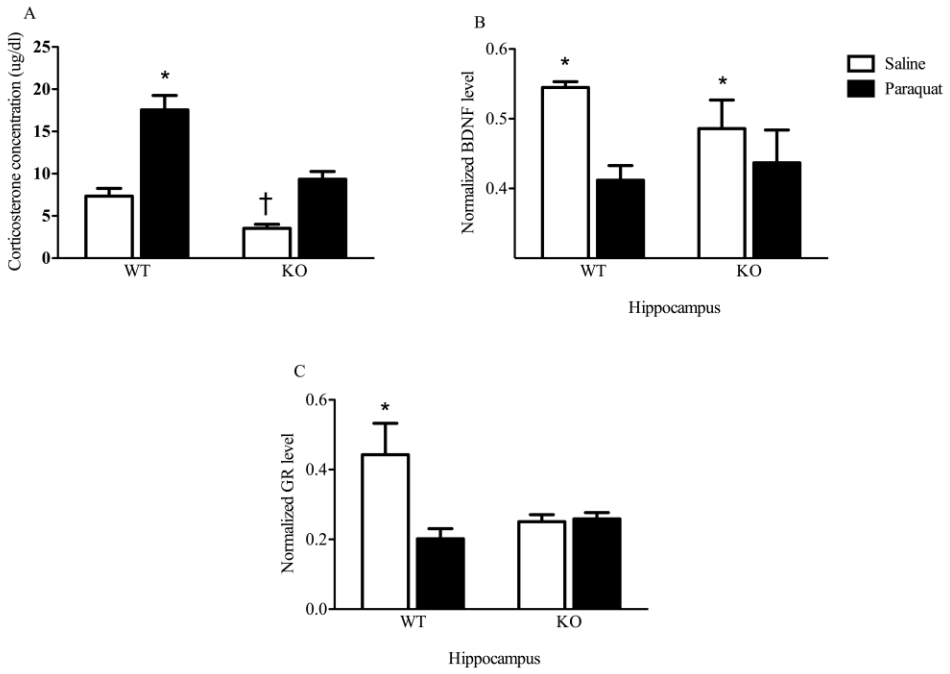

Figure 5: Paraquat induced elevated corticosterone levels that were blunted in LRRK2 null mice. LRRK2 ablation did not affect paraquat induced reduction in hippocampal BDNF or GR levels

Impact of LRRK2 on corticosterone levels, hippocampal brain derived neurotrophic factor (BDNF) and glucocorticoid receptor (GR) expression. Assessment of the principal stress hormone, corticosterone, revealed a paraquat induced elevation in wild type (WT) mice that was greatly blunted in LRRK2 knockout (KO) mice (* $p < 0.05$ relative to all other groups; panel A). At basal levels, saline treated KO animals also had lower levels of the hormone relative to their saline treated WT counterparts, as well as KO paraquat treated animals († $p < 0.05$ relative to all other groups). At the same time, while paraquat caused a reduction in hippocampal BDNF (panel B; * $p < 0.05$) and GR expression (Panel C), LRRK2 ablation did not alter these effects. However at basal levels LRRK2 ablated animals had lower expression of GR relative to saline treated WT animals (* $p < 0.05$ relative to all other groups). All data is expressed as mean ± SEM.

Effects of paraquat and LRRK2 ablation on plastic changes, oxidative stress, and markers of microglia activation in the nigrostriatal system

In order to observe whether these behavioral changes were linked to brain pathology, indices of oxidative stress, neuroplasticity, and "activation" state of phagocytic cells were also assessed primarily in the SNc. No significant changes were found for the catalytic NADPH oxidase (oxidative stress factor) gp91 subunit or the neuroplastic factor BDNF in the SNc (Figure 6 panel B and C respectively). However, the growth factor was reduced within the striatum of paraquat treated mice ($F(1,24) = 4.47$, $p < 0.05$), but was unaffected by LRRK2 ablation (Figure 6 panel A).

It was of interest to us to assess CX3CR1 and WAVE2 levels in the SNc. Elevated levels of these proteins can be used as markers of microglia activation. As such, we wanted to to assess whether paraquat exposure differentially alters expression of these proteins, and whether LRRK2 modulates this effect. In regard to WAVE2, we hypothesized that our paraquat dosing regimen would lead to elevated levels of the protein. This is because, when in a pro-inflammatory state (which is typically induced by paraquat), microglia require reorganization of the actin cytoskeleton, and, WAVE2 is important in regulating actin dynamics [270], [382]. Additionally, given our highly interesting behavioural findings demonstrating that LRRK2 KO protects against paraquat induced toxicity at the behavioral level, and findings showing that microglia responses are inhibited when LRRK2 is ablated from previous studies, we hypothesized that the paraquat provoked WAVE2 increase would be reversed in LRRK2 KO

animals. Accordingly, contrary to our hypothesis, a main effect of Injection was observed such that paraquat exposed animals had lower levels of WAVE2 relative to their saline exposed counterparts $F(1,19) = 5.021$, $p < 0.05$; Figure 36 panel E). However no Genotype X Injection effect was observed.

In regard to CX3CR1, based on the findings that microglia CX3CR1 signaling results in an anti-inflammatory phenotype of the glial cell [383], and that LRRK2 ablation likewise results in reduced secretion of pro-inflammatory molecules [3], we hypothesized (1) that paraquat exposure would lead to a reduction in CX3CR1 levels (indicative of a pro-inflammatory state) and (2) that increased levels of the fractalkine receptor would be shown in the SNc of LRRK2 KO mice, and perhaps even enhanced in paraquat exposed KO animals. Results of the two-way ANOVA demonstrated a main effect of Genotype such that KO animals had higher levels of the receptor relative to WT counterparts ($F(1,17) = 6.99$, $p < 0.05$; Figure 6 panel C). However, no Injection, or Genotype X Injection effect was observed.

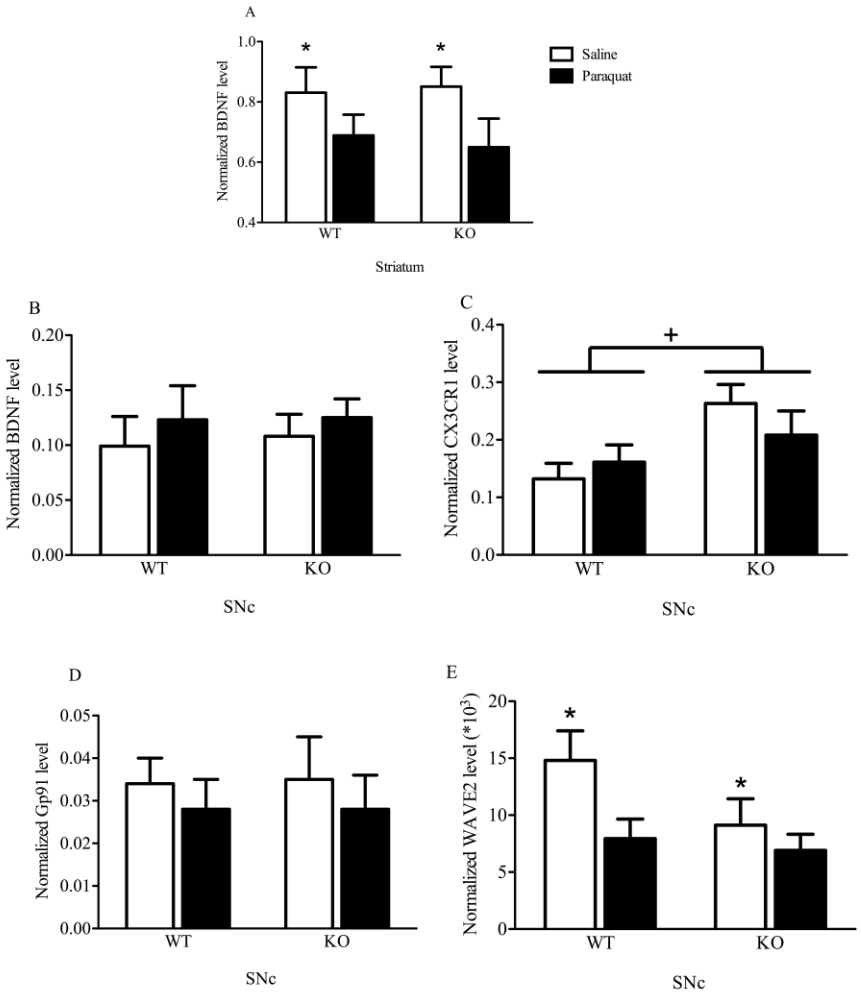

Figure 6: Paraquat induces changes in striatal BDNF and SNc WAVE2 levels. LRRK2 KO animals show higher levels of CX3CR1 in the SNc.

Impact of LRRK2 on nigrostriatal plasticity, oxidative stress, as well as microglia activation state in the SNc in paraquat treated mice. Paraquat caused a reduction in striatal (panel A) but not SNc BDNF (panel B) levels that was not prevented by LRRK2 ablation. While paraquat caused a reduction in WAVE2 expression levels in the SNc (panel E) it did not influence gp91 (panel D) or CX3CR1 levels (panel C). LRRK2 KO animals showed higher SNc levels of CX3CR1. * $p < 0.05$ relative to saline treated animals irrespective of genotype; + relative to WT animals irrespective of paraquat treatment. All data is expressed as mean ± SEM.

LRRK2 KO blunts paraquat induced organ weight alterations

As an index of any gross organ pathology, organ weight measures were taken (Figure 3). These yielded significant Genotype X Injection interactions for the liver ($F(1,32) = 6.381$, $p < 0.05$) and lungs ($F(1,32) = 4.369$, $p < 0.05$). In the liver, paraquat reduced organ weight in WT animals ($p < 0.05$; Figure 7 panel A) and LRRK2 KO prevented this effect. Similarly, the kidney displayed patterns of weight change identical to the liver (data not shown). In the lung, paraquat increased organ weight ($p < 0.05$) and again, this effect was prevented by LRRK2 ablation (Figure 6 panel B). The former effect could be related to liver atrophy, whereas the later effect could be attributable to LRRK2 preventing the expected infiltration of immune cells and pneumatic inflammation that characterizes paraquat's typical lung toxicity. Finally, no difference in spleen weight was evident (data not shown).

In parallel with the assessments of WAVE2 and CX3CR1 levels in the SNc, levels of these proteins were also analyzed in the liver and lungs (Figure 7). Accordingly, a main effect of genotype was observed for WAVE2 levels in the liver ($F(1,12) = 4.702$, $p < 0.05$). LRRK2 null mice displayed lower levels relative to their WT counterparts ($p < 0.05$; Figure 7 panel C). In contrast, no significant difference was observed for liver CX3CR1 levels. Within the lung, a significant Genotype X Injection interaction was observed for WAVE2 ($F(1,12) = 5.752$, $p < 0.05$). LRRK2 null mice had lower lung WAVE2 levels than WT mice ($p < 0.05$; Figure 7 panel D) and they had similar levels to paraquat treated animals. Again, there were no significant differences between the groups for lung CX3CR1 levels.

Finally, WAVE2 and CX3CR1 levels within the spleen were unaffected by the treatments or genotype; however, we did find differences in splenic immune cell populations (Figure 7 panel E). In accordance, with the important immune trafficking role of the spleen, we found a marked increase in CD68+ mononuclear cells (largely reflecting macrophages) in paraquat treated WT mice. However, this effect was largely prevented by LRRK2 KO (Figure 7 panel E).

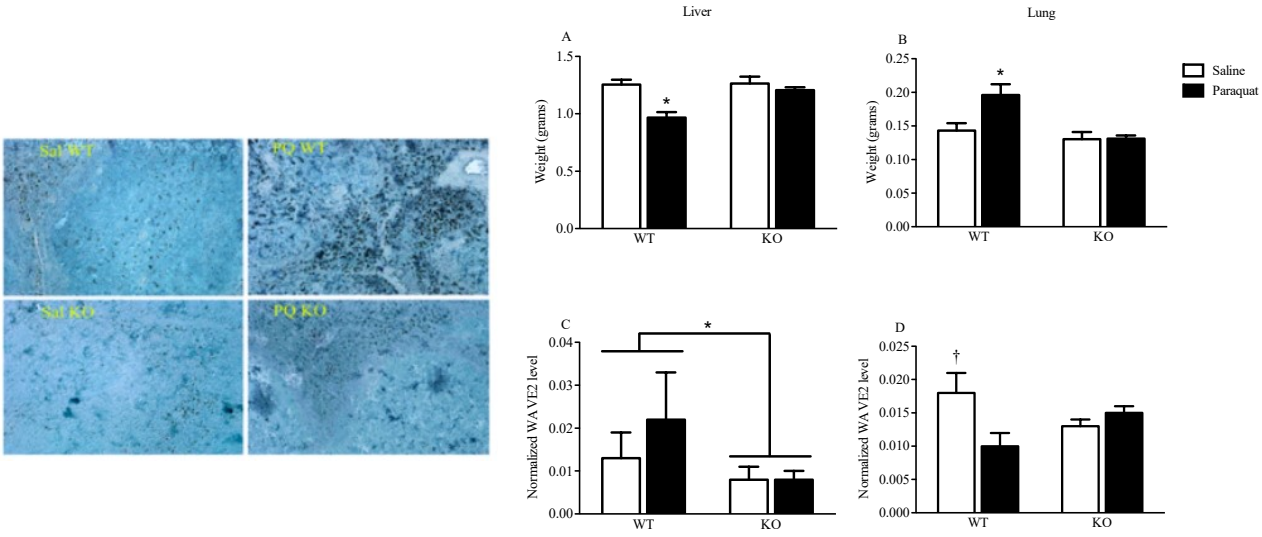

Figure 7: Paraquat induces alterations to peripheral organ weights and monocyte expression in WT but not LRRK2 KO mice.

Effects of LRRK2 in the context of paraquat treatment on peripheral organs of aged mice. Paraquat reduced liver weight (panel A), while increasing lung weight (panel B) in wild type (WT) mice. LRRK2 knockout (KO) totally prevented these effects. WAVE2 levels were reduced by LRRK2 KO in both the liver (panel C) and lungs (panel D). Further, paraquat also reduced WAVE2 in the lungs of WT, but not LRRK2 KO mice (panel D). Within the spleen, paraquat provoked increased CD68+ mononuclear immune cell infiltration (PQ WT), whereas LRRK2 prevented this immune cell accumulation (PQ KO). † or * $p < 0.05$ relative to all other groups; + $p < 0.05$ relative to WT mice irrespective of genotype. All data is expressed as mean ± SEM.

Discussion

Paraquat-induced neurotoxicity has many characteristics similar to idiopathic PD including motor and non-motor disturbances which accompany hallmark pathological features (i.e. microglia activation, Lewy body inclusions) of the disease [49], [65], [370]. Many of these characteristics become enhanced when the toxin is combined with other stressors (i.e. psychological, chemical, immune, aging) [46], [168], [171], [284]. Age itself can act as a factor that magnifies pathology, as the aging processes can reduced trophic support and lead to the manifestation of reactive gliosis, which can render already vulnerable SNc dopamine neurons more susceptible to further insults [384]. This is in line with the multiple-hit hypothesis of the disease which suggests insults throughout the course of one's lifetime can interact with age induced deficits to gives rise to sporadic forms of the disease [19].

In line with this notion, we and others have shown that the neurotoxic effects on the nigrostriatal system in paraquat exposed mice are enhanced both in the context of aging [370], with other chemical toxicants [168][75], as well as in mice who have been pre exposed to the endotoxin LPS [46], [171]. For instance, we previously showed that LPS + paraquat exposure induced motor behavior deficits and microglia mediated dopamine cell loss in the SNc which was greater than that induced by paraquat alone. The inflammatory related gene, LRRK2, may be important in this regard, as knocking out LRRK2 completely protected against the SNc cell loss and motor behavioral alterations induced by the toxins. In the current study, we were interested in whether or not LRRK2 is critical in LPS and paraquat pathology in older mice.

However, we unexpectedly found a significant degree of mortality in the older wild type mice, conversely LRRK2 null mice were resistant in this regard. As such, we re-formulated our research question, so as to ascertain whether LRRK2 might be important or general overall toxicity of paraquat in these older mice.

We found that after the 5th paraquat injection wild type mice displayed marked signs of sickness, but that this was completely absent in the LRRK2 knockouts. Similarly, LRRK2 ablation prevented the home cage and nest building behavioral deficits induce by the toxin. Of course, the motor deficits can be explained by the overall sickness profile, they might be related to the paraquat induced reduction in TH, which suggests that TH positive neurons in the SNc were also markedly reduced [385]. While not specifically addressed in the current study, paraquat exposure does lead to a reduction in SNc dopamine neurons in older mice (i.e. 8 months as opposed to 2 month) [379]. Whatever the case, our highly interesting data confirmed that LRRK2 plays a role in paraquat induced toxicity.

Recent studies have demonstrated that LRRK2 is an essential component in peripheral and central inflammatory responses [264], [380]. Indeed, LRRK2 is highly expressed by a variety of immune cell types, including macrophages, as well as in the brain resident immune cells microglia [380]. In fact, expression of LRRK2 levels in microglia are markedly higher than in SNc dopamine neurons [257] and LRRK2 helps to modulate the response of these cells to environmental insults [257], [276]. For example, LRRK2 inhibition results in impaired recruitment towards chemoattractant proteins, as well as a reduction and release of

inflammatory cytokines in response to LPS [257], [274], [276]. Accordingly, we were interested in whether or not LRRK2 knockout modulates CNS inflammatory environment (i.e. microglia) in response to toxic levels of paraquat, as indicated by two critical modulators of the cell; namely, WAVE2 and CX3CR1, as these are thought to be critical for microglial phenotype conversion to an active/inactive state [386]. Indeed, pharmacological and genetic ablation of these factors has been shown to greatly modify inflammatory response processes [387], [388].

It will be recalled that WAVE2 is critical regulator of the proinflammatory morphological changes that occur in microglia and macrophages, whereas CX3CR1 is more important in regulating anti-inflammatory responses. WAVE2 is a Rac1 effector molecule highly expressed on hematopoietic cells and microglia [352]. Its other two isoforms include WAVE1 found primarily in neurons and oligodendrocytes, and WAVE 3 expressed only in neurons [389]. Upon activation of immune recognition receptors, WAVE2 has been shown to be responsible for immune cell activation through promoting actin polymerization and thus, reorganization of the cytoskeleton to prepare the cell for activation, migration, motility, or phagocytosis [352]. In fact, members of our own group have very recently found WAVE2 induced phagocytic function to be critically regulated by LRRK2 [270]. Moreover, in previous findings from our lab (seen in the preceding chapter), we demonstrated the importance of WAVE2 in LRRK2's role in microglial reactivity to LPS. In this chapter, however, we found a different profile of WAVE2 levels, such that paraquat induced a reduction of WAVE2 levels in the SNc which was not modulated by LRRK2 ablation. This suggests that WAVE2 is having very different effects in the context of paraquat, as compared to LPS. However, it is important to underscore that we

presently assessed WAVE2 in whole SNc tissue, whereas in the previous chapter we specifically isolated microglia. Accordingly, given the limited expression of WAVE2 in neurons [389], it is possible in the paraquat exposed animals, the toxicant did impact neurons (and thus cytoskeleton reorganization) in addition to microglia, and that these alterations were masked by not isolating the specific cell type. As such, future studies should address the LRRK2-WAVE2 interaction important for microglia dependent response processes induced by paraquat.

In concert with WAVE2, we were also interested in looking at CX3CR1. Indeed, CX3CR1 is a receptor predominantly found on microglia and is strongly implicated in microglia regulation, through the promotion of anti-inflammatory factors upon interaction with its ligand fractalkine [390]. This ligand-receptor interaction is thought to maintain microglia in a surveillance state, but impedes these cells from releasing pro-inflammatory factors [391]–[394]. Recent evidence has shown that LRRK2 may impart some of its pro-inflammatory actions by inhibiting CX3CR1 [198], [358], [359], and we have shown an upregulation of CX3CR1 expression in LRRK2 knockout mice. Hence, we hypothesized that LRRK2 ablation might, in part, have beneficial effects by bolstering CX3CR1-dependent anti-inflammatory processes. Accordingly, consistent with previous findings, we found elevated levels of CX3CR1 in our LRRK2KO mice, yet, paraquat had no influence on CX3CR1 levels. It may be that the inherent basal elevation of this protein in knockouts is enough to protect against the toxic insult acting to keep the glia cells in an “anti-inflammatory” state.

In addition to the central effects of LRRK2 knockout, it was also of interest to assess peripheral signs of inflammation and pathology, as has been implicated in PD [395]–[397] [398], [399]. In fact, peripheral gram-negative bacterial infections were associated with PD and LRRK2 itself is mobilized by bacterial infection and contributes to the production of ROS from macrophages [337]. We presently found that LRRK2 knockout prevented the accumulation of CD68+ monocytes within the spleen in paraquat treated aged mice. Similarly, LRRK2 deficiency also prevented the changes in organ weight induced by paraquat, suggesting that LRRK2 knockout protected against organ toxicity. Furthermore, LRRK2 null mice also had reduced WAVE2 levels in the liver and lungs, consistent with a role for LRRK2 in peripheral phagocytic or general immune cell mobilization. In fact, these findings are in line with reports that paraquat damaged the lung, while altering macrophage levels and phagocytosis [400]–[402] and raise the possibility that LRRK2 was modulating the overall inflammatory tone in the body.

A final consideration of this study is whether LRRK2 can influence the general stress profile of toxicant treated mice, which of course, can also impact neuronal viability. Indeed, consistent with our previous findings [204], [283], [403], paraquat was able to act as a systemic stressor increasing plasma corticosterone, as well altering hippocampal glucocorticoid receptor and brain derived neurotrophic levels. Moreover, our paraquat dosing regimen induced sickness behavior and interfered with nesting behaviors. LRRK2 knockout prevented all of these deficits (apart from BDNF), suggesting its involvement in the immune-brain circuits crucial for prototypical illness (e.g. shivering, fever, piloerection, ptosis) and stressor-relevant

social species-specific behaviors. Indeed, hypothalamic brain nuclei are known to be exquisitely sensitive to treatments that provoke immune activation, and mediate the typical sickness profiles associated with such treatments [404]. Thus, LRRK2 appears to be important for stress related hormonal processes aligned with toxicological threats and sickness responses. In fact, our novel findings that LRRK2 had reduced basal levels of both corticosterone and the hippocampal glucocorticoid receptor suggest a possible link between LRRK2 and HPA activity, and might represent a mechanism links stress and the motor and co-morbid features of PD, however future research is needed in the area.

In summary, our study has confirmed a role for LRRK2 in paraquat induced toxicity in aged mice. Furthermore, our study suggests that LRRK2 may not only be important for neuroinflammatory and chemical alterations in the SNc, but may also be important for the sickness and hormonal responses to systemic paraquat administration. Our findings also extend beyond a PD diagnosis and potentially raise the possibility of using LRRK2 inhibitors against paraquat induced toxicity in human patients.

General Discussion

It is generally accepted that the emergence of Parkinson's disease (PD) results from the complex interaction between environmental insults and likely underlying genetic vulnerabilities [26], [28], [31]. Both epidemiological and experimental findings have specifically implicated pesticides, heavy metals, and infectious agents [33]. For instance, several large scale meta-analyses have implicated a variety of pesticides, including the commonly used herbicide, paraquat [405]. Also, in rodents, systemic injection of paraquat has been shown to dose-dependently damage the nigrostriatal system in conjunction with the appearance of motor deficits [370]. In line with the notion that PD stems from multiple hits with such insults over one's lifetime, a main goal of the current thesis was to evaluate the interactive or independent effects of different types of stressors (including psychological, immune, and aging) on the behavioral and biological PD related features induced by paraquat, in order to add to the literature and further our growing understanding in the area.

Chapter 1 of the current dissertation evaluated the combination of psychological stress with paraquat exposure, Chapter 2 investigated the impact of immune priming with the inflammatory agent LPS prior to toxin exposure, and Chapter 3 looked at the toxic effects of paraquat in mice older than what we typically use in our models. Overall, the experiments described in the current thesis demonstrate that the combination of different stressors with paraquat did influence symptom presentation as well as neurochemical alterations provoked by the toxin. Specifically, we found that paraquat provoked a loss of substantia nigra (SNc) dopamine neurons, and this effect was enhanced when the toxin was administered in conjunction with the inflammatory potentiator, LPS (Chapter 2). Likewise, we found that the age of exposure

influenced the damaging effects of the toxin (Chapter 4). However, the effects of paraquat on most non-motor behaviors or dopamine neuron survival specifically was not influenced by exposure to a chronic unpredictable stress (another risk factor implicated in PD) regimen, despite the combination influencing motor coordination (Chapter 1).

In addition to assessment of multi-toxicant hits and PD, a second aim of the current thesis was to investigate whether inflammatory mechanisms contribute to deleterious effects of these environmental hits. In this regard, we focused upon the gene, leucine rich repeat kinase 2 (LRRK2), given that it is not only a common PD-linked gene, but also the fact that its highest concentrations are found in immune cells [406]. In fact, many of the functions of LRRK2 involve modulation of the inflammatory immune system [251]. As such, we were interested in determining whether or not LRRK2 might be an important mediator of PD-like effects induced in our animal models as these models typically demonstrate involvement of neuro-immune processes [46], [291]. Accordingly, our studies (seen in Chapter 2 and 3) demonstrated the critical importance of LRRK2 in mediating the damaging effects of paraquat or animals primed with LPS before receiving the pesticide. In particular, using LRRK2 deficient mice in comparison to their wild type littermates, we show that LRRK2 ablation protects not only against the damaging effects of paraquat in our older animals, but also against the behavioral and microglia mediated dopamine neuron degeneration induced by paraquat or the LPS + paraquat combination. We also further added to the understanding of the role of LRRK2 by demonstrating that the protein was involved in modulating the phenotypic state of microglia. In particular, we found evidence that the actin regulatory protein, WASP-family verproline

homologous protein-2 (WAVE2), might have been fundamental for the cytoskeletal re-organization and adoption of an active appearance in LPS primed microglia, and that LRRK2 may regulate chemokine receptor 1 (CX3CR1) expression. Whatever the case, in these studies, we are the first to show the importance of LRRK2 in paraquat toxicity as well as in multi-hit models of PD.

From the present data, it is not entirely clear how LRRK2 is affecting dopaminergic neurotoxicity, however, we believe that four downstream mechanisms are of crucial importance (figure 1). As will be discussed in the ensuing sections, we believe that in response to inflammatory driven challenges LRRK2 can regulate: (1) microglia phenotype, (2) microglia-neuron interaction, (3) processes intrinsic to the local dopamine neuron itself, and (4) peripheral to central immune communication.

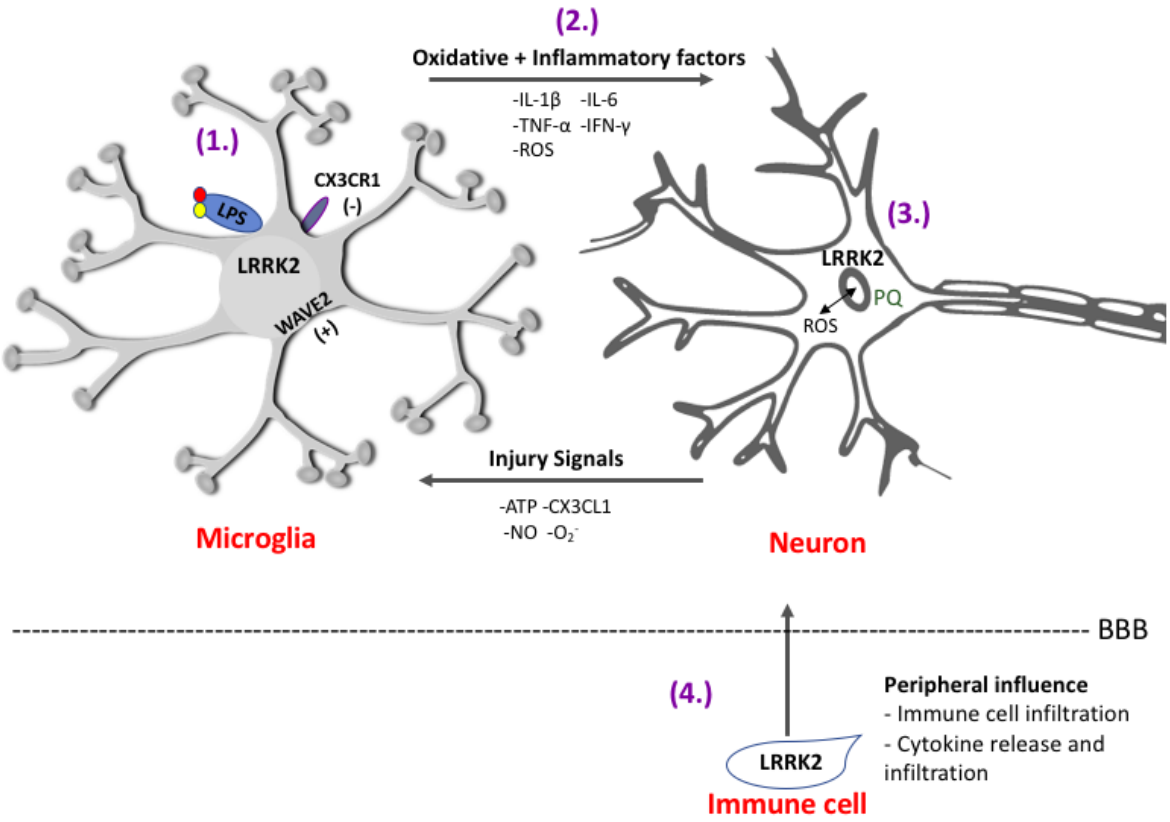

Figure 1: LRRK2 involvement in LPS, paraquat or LPS + paraquat toxicity

As depicted in the above diagram, LRRK2 can potentially regulate 4 important factors that may influence paraquat (PQ), LPS, or LPS + PQ toxicity. In response to PQ, LPS, or LPS + PQ LRRK2 can potentially regulate (1) microglia phenotype, (2) microglia-neuron interaction, (3) processes intrinsic to the local dopamine neuron itself, and (4) peripheral to central immune communication.

LRRK2 ablation regulates microglia phenotype (WAVE2 and CX3CR1)

It will be recalled that microglia play an important role in facilitating neurodegeneration within SNc dopamine neurons [128]. This occurs in part due to their ability to migrate and directly contact neurons through extendable processes and then either phagocytize them or produce proteases that damage neuronal membrane and cause rupture and necrotic death [3], [128]. As a general example, in response to immunological and environmental insults (such as those provoked by paraquat or LPS for example), these cells undergo a phenotypic shift that is characterized by the enhanced release of inflammatory and oxidative factors [129], [155]. This state of activity (or reactivity) essentially exists along a gradient ranging from resting to hyperactive with each state being principally controlled by a number of regulatory proteins [50]. Essentially, in a state of high activity (in response to different insult signals), microglia adopt a phenotype that is largely characterized by an increase in respiratory bursts and the release of these factors [50]. Here microglia adopt an amoeboid-like shape in which there is a retraction of their branch-like processes that normally extend from the cell body [50]. This is in stark contrast to a more "resting" phenotype of these cells, which is more associated with the release of neuroprotective and anti-inflammatory factors [50]. We and others have posited that toxin induced adoption of a more active phenotype will, over time, and via the influence of multiple "activating hits", promote nigrostriatal degeneration [19].

With regard to microglia, relative to other brain regions, the SNc has a particularly high concentration of these cells possibly rendering the area more vulnerable to various insults [166], [167]. It is important to note however that the exact role of these cells in neurodegeneration still remains a little unclear [19]. For instance, whether these cells play a primary role in the genesis

of the disease, or are secondarily activated in response to some primary pathology (i.e. secondary driving force of dopamine neurodegeneration), is still up for debate [19]. Whatever the case, the principal findings of the current thesis further add to the growing evidence that microglial-dependent processes are involved in PD, but also adds important new data suggesting that LRRK2 might be a critical regulator of glial and neurodegenerative outcomes elicited by different insults.

In any regard, in order for microglia to become "activated" actin cytoskeleton reorganization must occur along with upregulation of relevant proteins [407]. Importantly, LRRK2 has been shown to not only interact with actin itself, but also actin regulatory proteins known to regulate organization of the cytoskeleton [270], [271], [408]. As mentioned previously, one mechanism that aides cytoskeletal reorganization is via engagement of the WAVE2 pathway [270], [352]. Indeed, WAVE2 has been shown to promote cytoskeletal reorganization in response to LPS, which allows these cells to adopt an active appearance as well as engage in phagocytosis to clear the invading pathogen and/or cells potentially damaged by the endotoxin [270]. Notably, our research demonstrates the potential importance of a LRRK2-WAVE2 interaction in allowing microglia to adopt an active morphology, as LRRK2 ablation blunted the priming effects of LPS on microglia, which was characterized also by having reduced WAVE2 levels (Chapter 2). In fact, it has very recently been shown that the LRRK2-WAVE2 interaction allowing immune cells to adopt an active phagocytic phenotype is largely dependent on the kinase-mediated phosphorylation of WAVE2 by LRRK2 [270].

Indeed inhibiting LRRK2 kinase activity results in a reduction in WAVE2 phosphorylation and actin mediated phagocytosis in bone-marrow derived macrophages (BMDMs) [270].

Moving forward it becomes important to better understand this LRRK2-WAVE2 interaction as it pertains to microglia activation and PD. It is also important to understand how this interaction might vary as a function of the eliciting insult. In fact, while we found that LRRK2 knockout prevented the elevated WAVE2 levels of the prototypical TLR4 agonist, LPS, no such effect was observed when paraquat alone was the insult (Chapter 3). It may be then that LRRK2s ability to alter microglia response or mobility processes is dependent upon the type of receptor activated. Indeed, while it has been established that TLR4 stimulation on microglia increases LRRK2 phosphorylation, there are a number of other surface molecules present on these cells including those belonging to families of other pattern recognition receptors (PRRs), scavenger receptors, as well as cytokine and chemokine receptors [409]. These receptors can sequentially engage in the promotion of an alternatively activated morphology, and LRRK2 may or may not be involved. For instance, alterations in LRRK2 expression does not alter macrophage activity when stimulated with a TLR2 agonist, PAM_3CSK_4 [269]. Alternatively, LRRK2 was important for the inflammatory activation of macrophages in response to zymosan, a cell-wall component found in the flora of the gut, which acted through the c-type dectin-1 receptor [269]. As such, the role LRRK2 plays in neuroinflammation may be different and dependent on the unique microenvironment and signaling pathways associated with a particular insult [3].

Just as there are a number of signaling mechanisms that allow microglia to adopt a highly reactive state, there are also a number of endogenous regulatory mechanisms that maintain these cells in a homeostatic resting state [409]. Under basal conditions for example, anti-inflammatory cytokines, suppressor of cytokines (SOCs) signaling, and other inhibitory proteins, have all been shown to inhibit microglial conversion to an activated state [409]. One such important factor is the chemokine, fractalkine (CX3CL1) [410]. Expressed mainly in neurons, CX3CL1 interacts with its receptor, CX3CR1, which is exclusively expressed on microglia [383]. Normal ligand-receptor interaction of these molecules promotes dynamic motility processes of microglia, their migration, and their release of anti-inflammatory cytokines, as well as potentially trophic factors [410]. As mentioned in the general introduction, under stress conditions, the CX3CL1-CX3CR1 neuron-microglia crosstalk may lead to reduced activation of microglia in conjunction with the restricted release of neurotoxic factors, which of course would have beneficial outcomes [345], [394], [411], [412]. Indeed, it is well documented that CX3CR1 ablation results in a stark increase in microglia activity (characterized by an active appearance and augmented inflammatory cytokine expression) in response to challenge [345], [394], [411], [412]. For instance, in response to the PD relevant neurotoxin MPTP, CX3CR1 deficient mice show augmented levels of microgliosis (a high number of activated microglia) and nigrostriatal damage [345]. Likewise, CX3CR1 deficient mice overexpressing α-SYN^{A53T} show enhanced nigrostriatal degeneration and accompanying microgliosis as well as the restricted and potentiated expression of anti- and pro-inflammatory factors respectively [346]. Conversely, administration of CX3CL1 in neurotoxic models have

demonstrated protective outcomes [412], [413]. As an example, CX3CL1 overexpression attenuates SNc dopamine neurodegeneration provoked by α-SYN [413]. Likewise, 6OHDA rats treated with CX3CL1 show that the chemokine protects against the toxin induced nigrostriatal damage and microglia activity [412]. Whatever the case, it appears that CX3CL1/CX3CR1 signaling plays an important inhibitory role in regulating overactive microglial inflammatory response processes known to be involved in dopamine neuron degeneration.

As observed in Chapters 2 and 3 of this dissertation, we show that the beneficial effects of LRRK2 ablation in response to our environmental and endotoxin challenge, may be, in part, due to augmented CX3CL1-CX3CR1 dependent anti-inflammatory processes. Indeed, basal elevated CX3CR1 levels were observed in our knockout mice, and we showed that this occurred in conjunction with a reduction in an active microglia appearance in response to LPS (Chapter 2), as well as may be potentially important for paraquat induced toxicity (Chapter 3). Our findings are in agreement with others who also demonstrated that LRRK2 knockout mice have elevated microglia levels of CX3CR1 [414]. In fact, these researchers showed that while LRRK2 knockout prevented the impact of nigrostriatal injection of LPS, further CX3CR1 deficiency restored the impaired inflammatory response; hence, the lower levels of CX3CR1 essentially neutralized the LRRK2 effects [414].

Future research is required to address the mechanisms of exactly how LRRK2 may affect microglia CX3CR1 function or vice versa. For instance, it may be that LRRK2 imparts an

elevated inflammatory state by down-regulating CX3CR1 levels [265], [269], [414]. In line with this notion, LRRK2 has been shown to enhance the association of the transcription factor, nuclear factor of activated T-cell 1 (NFAT1), with its cytosolic repressor protein (NRON), which resulted in the blocking of NFAT1 interactions with nuclear genes in macrophages [265], [269]. This specifically resulted in the reduction of CX3CR1 transcription [414], [415]. Hence, involvement in NFAT1 nuclear translocation, may be one way that LRRK2 affects CX3CR1 levels [414]. Of course, we cannot ignore LRRK2's involvement in other intracellular processes which may influence CX3CR1 expression [414]. In fact, LRRK2 has been shown to partially regulate other factors which may influence protein expression including endoplasmic reticulum-to-Golgi transport [251], [416], protein stabilization [417], and autophagy [263], [418]. Whatever the case, when taken together, our results demonstrate that LRRK2 may be important for regulating the microglia phenotype including their active appearance in response to LPS and endotoxin + paraquat exposed mice, as well as other factors involved in appropriately responding to different insults.

LRRK2 regulation of microglia-neuron communication

LRRK2 and cytokine release

In line with the notion that LRRK2 regulates microglia phenotype, it is also very likely that LRRK2 may regulate factors that are involved in microglia-neuron communication including: (1) the release of soluble factors from microglia known to influence neuronal integrity and survival, or (2) processes involved in cell to cell contact such as phagocytosis. In

regard to the former, it is well documented that excessive and prolonged release of oxidative factors by these cells can cause damage to a plethora of biomolecules (i.e. lipids, proteins, DNA) eventually leading to cell death [208]. Additionally microglia release of pro-inflammatory cytokines can not only further recruit and activate other immune cells, but also trigger pro-death pathways [208]. As an example, IFN-γ activation of the IFN-γ receptor complex l-α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) GluR1 on neurons can result in activation of cell death pathways by increasing intracellular calcium influx and glutamate neurotoxicity [419]. Also, activation of the neuronal p55 receptor by TNF-α can silence intracellular survival signals and caspase-dependent apoptotic cell death [210].

Notably recent evidence suggests that LRRK2 may influence transcription factors relevant for cytokine and chemokine expression [3]. Of course, this is in part supported by our own research demonstrating that LRRK2 ablation resulted in elevated expression of the anti-inflammatory associated receptor CX3CR1, but also by others demonstrating ability of LRRK2 to regulate transcription factors relevant for pro-inflammatory cytokine release [265], [269], [420]. For instance, in microglia, LPS induced LRRK2 expression was associated with augmented NF-κB dependent transcription of pro-inflammatory cytokines [257], [265], [267]. In contrast, silencing LRRK2 attenuated NF-κB transcription, as well as the downstream expression of iNOS and COX-2, along with IL-6, TNF-α, and IL-1β in response to the endotoxin [257], [265], [267]. Accordingly, it may also be that LRRK2 regulates the release of trophic and other anti-inflammatory factors in response to insult, which in turn would favor neuronal survival. In our current studies, while we did not see elevated levels of BDNF in the

SNc in response to paraquat in our LRRK2 knockout animals, we cannot reject the possible contribution of other trophic factors including glial derived neurotrophic factor (GDNF), nerve growth factor (NGF) , neurotrophin (NT)-3, or even possibly those derived from astrocytes such as fibroblast growth factor-2 (FGF2) [421]–[425]. Indeed, while the role of astrocytes were overlooked in the current thesis, we cannot ignore the highly complex interplay between astrocytes, microglia, and neurons in our models [426].

As an interesting aside, evidence suggests that LRRK2 may play different roles in regard to cytokine release in peripheral (macrophages) versus central (microglia) immune cells in response to diverse insults (for a review see Lee et al., 2017 [3]). For instance, while LRRK2 ablation is shown to attenuate the release of pro-inflammatory factors in response to TLR4 activation by LPS in microglia [257], [265], [267], no such effects are observed in LPS stimulated BMDMs [261], [268], [427]. This suggests that, in contrast to microglia, in peripheral macrophages, LRRK2 does not appear to be critical for TLR4 response stimulated cytokine release, but instead, raises the interesting possibility that LRRK2 may modulate responses to other immune stimulating agents relevant to PD such as alpha synuclein, or even perhaps environmental toxins including paraquat [3], [428]. Indeed, as will be discussed later there is support for involvement of LRRK2 in peripheral cytokine activity in the pathogenesis of PD. For instance, both asymptomatic and PD patients carrying the G2019S mutation display elevated levels of pro-inflammatory cytokines [396]. Moreover, abolishing LRRK2 in BMDMs alters cytokine expression in response to zymosan a TLR2 receptor agonist [269].

LRRK2 and Phagocytosis

While primarily associated with the clearance of apoptotic cells or invading pathogens, overactivation of phagocytic microglia has also been associated with enhanced neuronal death and damage [429]. This is supported by the fact that, while there is ongoing microglia activation in the SNc throughout the course of the disease, this active phenotype also displays phagocytic characteristics [430]. Moreover, preventing microglia phagocytosis has been shown to diminish LPS, amyloid beta, and rotenone provoked neuronal death [431]–[435].

Notably, in addition to regulating cytokine release, LRRK2 may also be involved in regulating phagocytic processes [270], [272]. LRRK2 interacts with phagosomes and other actin regulatory proteins involved in cytoskeletal re-organization (i.e. WAVE2 discussed above) [270], [271], [408]. As well, blunted phagocytic uptake of bioparticles was evident with LRRK2 ablation, or inhibiting its kinase activity [270], [272]. For instance, in response to the Human Immunodeficiency Virus (HIV)-1 trans activator (Tat) protein, immortalized microglia cells display impaired engulfment of latex beads following LRRK2 kinase inhibition [272]. Moreover, both basally and in response to various phagocytic inducers (including LPS, polyI:C, and zymosan), LRRK2 null microglia display impaired phagocytosis of *Escherichia coli* bioparticles or latex beads, while conversely G2019S knockin microglia show enhanced phagocytic engulfment of these factors [270]. Similarly, in G2019S BMDM and primary dopamine neuron co-cultures, the loss of dopamine neurons has been attributed to impaired phagocytic actions rather than the release of oxidative-inflammatory factors [270]. Given our

findings from Chapter 2; namely, that LRRK2 ablation inhibits the LPS induction of the known phagocytic regulator WAVE2, it may also be that LRRK2 regulation of phagocytosis impacts LPS + paraquat neuronal death. To our knowledge, no studies have directly looked at whether paraquat, or LPS + paraquat stimulated LRRK2 null or mutant microglia impact midbrain dopamine neurons due to altered phagocytosis, as such this might be a fruitful avenue to explore.

LRRK2 involvement in the local dopamine neuron

It should be underscored that there is also the distinct possibility that the neuronal protection observed in our models was not from the altered microglia phenotype alone. Indeed, although not the primary focus of the current studies, another pathway through which LRRK2 may be affecting toxicity is through inherent proteins within the dopamine neurons themselves. For instance, it is possible that LRRK2 is involved in regulation of processes that govern the detoxification of paraquat. In fact, in relation to peripheral tissue, in the brain, paraquat has a relatively prolonged half-life of up 1 month, which contributes to its accumulation with repeated doses [295], [369]. Given its prolonged half-life in CNS tissue, this suggests that the toxin is not metabolized efficiently, or possibly that it takes a relatively long time to expel the toxin. As such, the accumulation of intact paraquat overtime can result in the excessive generation of oxidative stress causing extensive and prolonged damage to neurons and the direct or indirect downstream activation of a number of neuronal pro-death pathways [295], [369]. Accordingly, there is a distinct possibility that LRRK2 impacts levels of reactive oxygen

species (ROS) in dopamine cells in response to paraquat, thus leading to the damaging effects of the toxin. Indeed, it has been demonstrated that levels of intracellular ROS are increased in dopamine cells encompassing a G2019S mutation which is further enhanced by external exposure to the ROS hydrogen peroxide, although the mechanisms leading to elevated levels of ROS are not yet understood [436]. In our current study (observed in Chapter 2) we demonstrate that endotoxin exposure could potentially alter dopamine machinery (i.e. dopamine transporter levels), and thus oxidative potential, and LRRK2 may be important in this regard.

While beyond the scope of the current thesis, LRRK2 has been linked to a plethora of other intra-neuronal signalling events involving direct protein-protein interaction, enzymatic activity, and events which occur indirectly as a result of these processes, which can impact neuronal function and survival in response to our models [437]. For instance, LRRK2 signalling has been shown to influence autophagic processes involved in the clearance of damaged organelles, such as impaired mitochondria or endoplasmic reticulum, as well as in the elimination of intracellular pathogens [437]. Given that paraquat can disrupt and/or damage multiple cellular processes including those related to mitochondrial and endoplasmic reticulum function [61], [63], LRRK2 may potentially be involved in mediating some of these functions. Likewise LRRK2 has been linked to other process that may influence neurotoxicity including vesicular transport, endo/exocytosis, as wells as protein transcription [437].

LRRK2 and peripheral immune influences

As might be apparent from preceding sections, in addition to its widespread expression in the brain, LRRK2 is also detected in a variety of peripheral tissues, most notably the liver, kidney, heart, and lungs [258], [438], [439]. Moreover, recent and emerging evidence suggests a prominent role for LRRK2 in peripheral immune cells as relatively high levels are observed in circulating and tissue-resident cells including monocytes, B-lymphocytes, and macrophages [258], [261], [440]. In fact, it appears that LRRK2 acts as a crucial mediator of peripheral inflammatory processes triggered by different environmental insults. In line with this notion, as observed in Chapter 3, we demonstrate that LRRK2 may play a role in peripheral inflammatory processes triggered by paraquat. In particular, we showed that paraquat exposure induced an "inflammatory profile" characterized by induced mobilization and accumulation of splenic monocytes, marked sickness behavior, plasma corticosterone elevations, and organ weight alterations, all of which were greatly ablated in our LRRK2 null animals.

There are a number of mechanisms through which LRRK2 might act to modulate peripheral immune responses [3]. In fact, similar to microglia, it appears that LRRK2 is involved in regulating pertinent inflammatory processes of these cells including mediating intracellular inflammatory signalling pathways (e.g., NF-κB) [267], influencing cytokine production [266], phagocytosis [270], and chemotactic responses in response to challenge [257]. Accordingly, while we demonstrate that LRRK2 ablation influenced paraquat induced mobilization and accumulation of peripheral immune cells, as mentioned above, it may also LRRK2 influenced cytokine release from these cells [3]. Notably, previous studies from our own lab have demonstrated that paraquat exposure can provoke alterations in peripheral

circulating cytokine levels [283], and that pro-inflammatory cytokines (most notably IFN- γ) are important for paraquat induced neurodegeneration [291]. In fact, IFN-γ has been shown to induce LRRK2 expression in peripheral immune cells including circulating macrophages [441] and CD14+ and CD16+ monocytes [262], in addition to microglia [266]. It may be then that paraquat provoked IFN-γ activity influences LRRK2 activity which in turn could lead to the exacerbated inflammatory induced damaging effects of the toxin.

Intriguingly, it is well known that peripherally derived cytokines can directly or indirectly influence CNS functioning, and be critical players in neurodegeneration. Owing to their relatively large size, cytokines do not readily cross the blood brain barrier (BBB) and thus rely on other means to gain entry into the central environment, or to impact CNS function [442]. Infiltration of cytokines can occur in areas lacking a fully functional BBB such as at the circumventricular organs (CVOs; e.g. the median eminence) [132], through saturable carrier-mediated transport mechanisms [132], or via functional lymphatic vessels that line the dural sinuses [79], [443]. Furthermore, cytokine influence on the CNS does not always require BBB permeation [444]. Indeed, cytokines interact with endothelial cells that line the interior of blood vessels of the BBB resulting in further production of these immune-messengers that spread throughout the brain parenchyma [180]. In fact, cytokine interaction with brain vasculature may also enhance BBB permeability by contributing to its breakdown, or even modifying tight junction proteins [444]. This interaction allows for the infiltration of not only immune factors (i.e. pro-inflammatory cytokines and immune cells), but also other potentially toxic factors (i.e. environmental factors) [444]. As an example, IFN-γ expression induced by the rabies virus

(RABV) has been shown to down regulate the tight junction proteins of brain microvasculature, allowing for the infiltration of immune cells into the CNS to help clear the virus [445]. While TNF-α induction has also been shown to enhance P-glycoprotein transporter expression, allowing for greater infiltration of xenobiotics and in turn inflammatory immune messengers [446], [447]. Cytokines may also influence central processes through their ability to activate various afferent neural pathways such as via vagus nerve stimulation [448]. Here, sensory vagal afferent fibers are able to detect elevations in cytokines and essentially relay this message to the brain of the peripheral immune state, as well as promote a central inflammatory environment [448]. In fact, this communication pathway is one that is highly important in production of febrile responses, as well as in reducing peripheral inflammation [448]. Whatever the case, LRRK2 regulation of peripheral cytokine activity can impact the state of the CNS environment and thus neuronal fate.

It is well known that peripheral immune cells along with other pro-inflammatory factors (i.e. cytokines) infiltrate the brain to contribute to the neurodegenerative cascade in response to challenge [134]. This is supported by the fact that, PD patients have altered serum expression of pro (i.e. TNF-α, IL-1β, IL-12-p40, FABP) and anti (IL-4 and IL-10) inflammatory factors [449], [450]. Moreover, post mortem analysis of PD brains demonstrate peripheral immune cell infiltration, with macrophages and T cells being observed in the nigrostriatal pathway [142], [451], [452]. It is thought that the infiltration of peripheral immune cells in these regions exacerbates the already existing microglia mediated neuroinflammatory toxic cascade [453], but some have alternately raised the possibly that these cells might be attempting to promote

repair of damaged and surrounding tissue [134], [454]. In support of the former, different treatments used to protect against toxin induced degeneration in PD animal models shows that the neuroprotection is coupled with a reduction of microglia, but also T cell infiltration into the midbrain [145], [146]. In fact, it has also been proposed that CD8+ cytotoxic T cells may actually kill SNc dopamine neurons, not only through interaction with the resident cells of the CNS, but also directly through cell to cell contact [146]. Indeed, in opposition to the ventral tegmental area (VTA), SNc dopamine neurons express the MHC I molecule in the presence of activated microglia, rendering these midbrain neurons susceptible to degeneration mediated by these T-cells [143]. It is important to note here that the neuroprotective effects of LRRK2 knockout observed in Chapter 2 may have been, at least in part, due to inhibition of peripheral cytokine activity, or even peripheral immune cell infiltration into the SNc. One caveat in our current studies is that we did not differentiate between local microglia and peripheral immune cells (including those of myeloid origin such as macrophages and monocytes). This raises the distinct possibility that peripheral immune cells with an active inflammatory phenotype contributed to SNc cell death, and that, LRRK2 may have been important in this regard. Whatever the case, LRRK2 regulation of peripheral immune cell mobilization, activation, and cytokine activity may have been important in our endotoxin and environmental challenge models.

Paraquat acts as a systemic stressor: potential involvement of LRRK2?

In chapter 3 of the current dissertation we demonstrated that LRRK2 ablation protected against the "sickness profile" induced by the pesticide in aged mice. We found evidence that strongly support the notion that paraquat acts as a systemic stressor influencing plasma corticosterone expression and hippocampal neurochemical activity (including monoamines, BDNF and glucocorticoid receptor levels). Moreover, consistent with the fact that it widely disburses throughout the brain, paraquat has also been shown to alter neurotransmitter activity in other stress sensitive regions including the locus coeruleus, paraventricular nucleus of the hypothalamus, nucleus accumbens, dorsal striatum, and central amygdala [283]. Intriguingly, the LRRK2 ablation of paraquat provoked increase in corticosterone and reduction of hippocampal glucocorticoid receptor suggests a potential role for LRRK2 in mediating HPA activity. However, alternatively the blunted corticosterone levels observed in our LRRK2 knockout animals may have actually been secondary to the diminished toxicity and inflammatory effects evident in the knockouts.

Accordingly, future studies should aim to disentangle the toxicant induced sickness and toxicity effects ascribed to LRRK2 from the impact of the protein upon neuronal and glial signalling parameters. Taking this into consideration along with the fact that LRRK2 is a very large and complex protein, we predict that its effects will be wide ranging likely involving many differing cell types, and impacting a wide range of functions. Moreover, it is also possible that its effects could change over the course of illness; for example, initially mobilizing immune cells to combat an environmental challenge, but over time these cells could interact with brain microglia and actually orchestrate neuronal damage. At the same time, given that G2019S

LRRK2 mutants display marked changes in dopaminergic neurotransmission [234], LRRK2 could be causing local network wide alterations in neurochemical signaling in addition to its impact on processes directly aligned with toxicity.

LRRK2 inhibition as a therapeutic approach?

The data presented in Chapter 2 and 3 of the current thesis support our notion that overactive inflammatory processes may, in part, contribute to the behavioral and biological outcomes provoked by LPS, paraquat, or LPS + paraquat. Moreover our data suggest that LRRK2 may also contribute to some non-motor features of the disease perhaps through altering not only inflammatory measures, but also possibly stress response hormones. Accordingly, it is tempting to say that our results support the proposition that LRRK2 inhibitors may be useful tools in the clinical domain, conceivably providing neuroprotection, while at the same time positively impacting motor and non-motor behaviors [455], [456]. However, while recently a number of LRRK2 inhibitors have been developed, specifically silencing LRRK2 may not be the ideal approach [455], [456]. There is growing concern, not only around the immunological outcomes, but other biological changes that may be imparted when using small molecule LRRK2 inhibitors long-term [455], [456].

Most of what is known about LRRK2 inhibition comes from *in vivo* and *in vitro* models in which expression has either been reduced (i.e. through RNA interference) or knocked out entirely, as opposed to clinical trials [456]. While a number of these studies have illustrated positive outcomes in the form of protection, they have also have brought to light potential

liabilities [455], [456]. For instance, LRRK2 knockout and/or kinase inhibition has demonstrated protection from LPS exposure [164], autoimmune uveitis [427], rhabdomyolysis-induced kidney injury [457], and HIV-Tat exposure [273]. Alternatively, while silencing LRRK2 in rodents does not appear to alter the overall neurobehavioral phenotype [458]–[460], there is relatively new evidence to suggest that it can cause age-dependent phenotypic changes in peripheral organs [439], [461], as well as alter endo/exocytotic and lysosome machinery [462]. Our research (and others) presented herein also shows that LRRK2 ablation can impact immunological responses, which, we cannot deny, could be potentially detrimental in the face of certain prolonged or acute challenge [456]. Moreover, given that PD is a prolonged progressives disorder, it is important to understand the effects of LRRK2 inhibition long-term, and to define whether or not there is a crucial therapeutic window in which to apply the inhibitors in order to achieve the best possible outcome while limiting negative side effects [456]. Whatever the case, this research expresses a strong need to further understand LRRK2 involvement, not only in immune response processes, but also other biological processes. Further understanding of LRRK2, its intracellular interactors, substrates and pathways may raise the possibility of a novel therapeutic approach not only for PD, but in other immune related diseases [456].

Conclusion

The findings outlined in this thesis demonstrate that the combination of different stressors (i.e. psychological, immune, and aging) with paraquat influence biological and symptom

presentation. While psychological stress exposure itself did not alter paraquat provoked microglia activation and death of SNc dopamine neurons similar to an immunological challenge (i.e. LPS), it did augment motor coordination behavior, and potentially some non-motor symptoms. Moreover age of exposure impacted the negative effects of the toxin. Our findings also support a role for LRRK2 in LPS, paraquat, or LPS + paraquat induced toxicity. Here we showed that LRRK2 ablation protects against the general "toxic" profile induced by paraquat in aged mice. Indeed, LRRK2 ablation altered the impact of the toxin on immunological, hormonal, and behavioural outcomes. Moreover we demonstrated that LRRK2 ablation also protects against the toxin induced dopamine cell loss in the substantia nigra and motor behavioral outcomes. In fact, this outcome was also demonstrated in a multi-hit model in which animals received an immunological challenge (i.e. LPS) prior to paraquat exposure. We showed that, in response to LPS, or the endotoxin + environmental toxin challenge, LRRK2 may play an important role in mediating microglia response processes that favor an enhanced inflammatory state which can be damaging to dopamine neurons. Accordingly, we suggest that further exploring LRRK2 signaling in response to LPS, paraquat, or LPS + paraquat, may hold substantial clinical promise in regard to the debilitating motor and even non-motor behaviors observed in PD.

References

[1] H. Sanjari Moghaddam, A. Zare-Shahabadi, F. Rahmani, and N. Rezaei, "Neurotransmission systems in Parkinson's disease.," *Rev. Neurosci.*, Mar. 2017.

[2] M. C. de Rijk, L. J. Launer, K. Berger, M. M. Breteler, J. F. Dartigues, M. Baldereschi, L. Fratiglioni, A. Lobo, J. Martinez-Lage, C. Trenkwalder, and A. Hofman, "Prevalence of Parkinson's disease in Europe: A collaborative study of population-based cohorts. Neurologic Diseases in the Elderly Research Group.," *Neurology*, vol. 54, no. 11 Suppl 5, pp. S21-3, 2000.

[3] H. Lee, W. S. James, and S. A. Cowley, "LRRK2 in peripheral and central nervous system innate immunity: its link to Parkinson's disease.," *Biochem. Soc. Trans.*, 2017.

[4] N. Eriksen, A. K. Stark, and B. Pakkenberg, "Age and Parkinson's disease-related neuronal death in the substantia nigra pars compacta," *Journal of Neural Transmission, Supplementa*, no. 73. pp. 203–213, 2009.

[5] P. Damier, E. C. Hirsch, Y. Agid, and A. M. Graybiel, "The substantia nigra of the human brain: II. Patterns of loss of dopamine-containing neurons in Parkinson's disease," *Brain*, vol. 122, no. 8, pp. 1437–1448, 1999.

[6] J. B. Schulz and B. H. Falkenburger, "Neuronal pathology in Parkinson's disease," *Cell and Tissue Research*, vol. 318, no. 1. pp. 135–147, 2004.

[7] M. Horstink, E. Tolosa, U. Bonuccelli, G. Deuschl, A. Friedman, P. Kanovsky, J. P. Larsen, A. Lees, W. Oertel, W. Poewe, O. Rascol, and C. Sampaio, "Review of the therapeutic management of Parkinson's disease. Report of a joint task force of the European Federation of Neurological Societies (EFNS) and the Movement Disorder Society-European Section (MDS-ES). Part II: Late (complicated) Parkinson's dise," *European Journal of Neurology*. 2006.

[8] H. Braak, K. Del Tredici, U. Rüb, R. A. I. De Vos, E. N. H. Jansen Steur, and E. Braak, "Staging of brain pathology related to sporadic Parkinson's disease," *Neurobiol. Aging*, vol. 24, no. 2, pp. 197–211, 2003.

[9] H. Braak, E. Ghebremedhin, U. Rüb, H. Bratzke, and K. Del Tredici, “Stages in the development of Parkinson’s disease-related pathology,” *Cell and Tissue Research*, vol. 318, no. 1. pp. 121–134, 2004.

[10] K. Wakabayashi, K. Tanji, S. Odagiri, Y. Miki, F. Mori, and H. Takahashi, “The Lewy Body in Parkinson’s Disease and Related Neurodegenerative Disorders,” *Molecular Neurobiology*, pp. 1–14, 2012.

[11] L. A. Volpicelli-Daley, K. L. Gamble, C. E. Schultheiss, D. M. Riddle, A. B. West, and V. M.-Y. Lee, “Formation of alpha-synuclein Lewy neurite-like aggregates in axons impedes the transport of distinct endosomes,” *Mol. Biol. Cell*, 2014.

[12] A. S. Harms, S. Cao, A. L. Rowse, A. D. Thome, X. Li, L. R. Mangieri, R. Q. Cron, J. J. Shacka, C. Raman, and D. G. Standaert, “MHCII is required for α-synuclein-induced activation of microglia, CD4 T cell proliferation, and dopaminergic neurodegeneration.,” *J. Neurosci.*, vol. 33, no. 23, pp. 9592–600, 2013.

[13] C. Hoenen, A. Gustin, C. Birck, M. Kirchmeyer, N. Beaume, P. Felten, L. Grandbarbe, P. Heuschling, and T. Heurtaux, “Alpha-Synuclein Proteins Promote Pro-Inflammatory Cascades in Microglia: Stronger Effects of the A53T Mutant,” *PLoS One*, vol. 11, no. 9, p. e0162717, Sep. 2016.

[14] A. De Virgilio, A. Greco, G. Fabbrini, M. Inghilleri, M. I. Rizzo, A. Gallo, M. Conte, C. Rosato, M. Ciniglio Appiani, and M. de Vincentiis, “Parkinson’s disease: Autoimmunity and neuroinflammation,” *Autoimmunity Reviews*, vol. 15, no. 10. pp. 1005–1011, 2016.

[15] S. Amor, L. A. N. Peferoen, D. Y. S. Vogel, M. Breur, P. van der Valk, D. Baker, and J. M. van Noort, “Inflammation in neurodegenerative diseases - an update,” *Immunology*, 2014.

[16] N. Pessoa Rocha, H. J. Reis, P. Vanden Berghe, C. Cirillo, N. P. Rocha, H. J. Reis, P. Vanden Berghe, C. Cirillo, N. Pessoa Rocha, H. J. Reis, P. Vanden Berghe, and C. Cirillo, “Depression and Cognitive Impairment in Parkinson’s Disease: A Role for Inflammation and Immunomodulation?,” *Neuroimmunomodulation*, vol. 21, no. 2–3,

pp. 88–94, 2014.

[17] M. Rentzos, C. Nikolaou, E. Andreadou, G. P. Paraskevas, A. Rombos, M. Zoga, A. Tsoutsou, F. Boufidou, E. Kapaki, and D. Vassilopoulos, “Circulating interleukin-15 and RANTES chemokine in Parkinson’s disease,” *Acta Neurol. Scand.*, vol. 116, no. 6, pp. 374–379, 2007.

[18] L. Klingelhoefer and H. Reichmann, “Parkinson’s disease as a multisystem disorder,” *J. Neural Transm.*, pp. 1–5, 2017.

[19] D. Litteljohn, E. Mangano, M. Clarke, J. Bobyn, K. Moloney, and S. Hayley, “Inflammatory mechanisms of neurodegeneration in toxin-based models of Parkinson’s disease.,” *Parkinsons. Dis.*, vol. 2011, p. 713517, 2010.

[20] C. H. Hawkes, K. Del Tredici, and H. Braak, “A timeline for Parkinson’s disease,” *Parkinsonism and Related Disorders*, vol. 16, no. 2. pp. 79–84, 2010.

[21] H. Bernheimer, W. Birkmayer, O. Hornykiewicz, K. Jellinger, and F. Seitelberger, “Brain dopamine and the syndromes of Parkinson and Huntington Clinical, morphological and neurochemical correlations,” *J. Neurol. Sci.*, 1973.

[22] A. Toulouse and A. M. Sullivan, “Progress in Parkinson’s disease-Where do we stand?,” *Progress in Neurobiology*. 2008.

[23] D. Sulzer, “Multiple hit hypotheses for dopamine neuron loss in Parkinson’s disease,” *Trends in Neurosciences*, vol. 30, no. 5. pp. 244–250, 2007.

[24] C. Klein and A. Westenberger, “Genetics of Parkinson’s Disease,” *Cold Spring Harbor Perspectives in Medicine*, vol. 2, no. 1. Jan-2012.

[25] M. Deleidi and T. Gasser, “The role of inflammation in sporadic and familial Parkinson’s disease,” *Cell Mol Life Sci*, vol. 70, 2013.

[26] B. R. Ritz, K. C. Paul, and J. M. Bronstein, “Of Pesticides and Men: a California Story of Genes and Environment in Parkinson’s Disease.,” *Curr. Environ. Heal. reports*, vol. 3, no. 1, pp. 40–52, 2016.

[27] P.-C. Lee, O. Raaschou-Nielsen, C. M. Lill, L. Bertram, J. S. Sinsheimer, J. Hansen, and B. Ritz, “Gene-environment interactions linking air pollution and inflammation in

Parkinson's disease," *Environ. Res.*, vol. 151, pp. 713–720, 2016.

[28] B. C. Jones, X. Huang, R. B. Mailman, L. Lu, and R. W. Williams, "The Perplexing Paradox of Paraquat: the Case for Host-Based Susceptibility and Postulated Neurodegenerative Effects," *Journal of biochemical and molecular toxicology*, vol. 28, no. 5. pp. 191–197, May-2014.

[29] J.-W. W. Lee and J. R. Cannon, "LRRK2 mutations and neurotoxicant susceptibility," *Exp. Biol. Med.*, vol. 240, no. 6, pp. 752–759, Jun. 2015.

[30] J. M. Biernacka, S. J. Chung, S. M. Armasu, K. S. Anderson, C. M. Lill, L. Bertram, J. E. Ahlskog, L. Brighina, R. Frigerio, and D. M. Maraganore, "Genome-wide gene-environment interaction analysis of pesticide exposure and risk of Parkinson's disease," *Parkinsonism Relat. Disord.*, 2016.

[31] J. R. Cannon and J. T. Greenamyre, "Gene-environment interactions in Parkinson's disease: Specific evidence in humans and mammalian models," *Neurobiology of Disease*, vol. 57. pp. 38–46, 2013.

[32] K. C. Paul, J. S. Sinsheimer, S. L. Rhodes, M. Cockburn, J. Bronstein, and B. Ritz, "Organophosphate Pesticide Exposures, Nitric Oxide Synthase Gene Variants, and Gene-Pesticide Interactions in a Case-Control Study of Parkinson's Disease, California (USA)," *Environ. Health Perspect.*, vol. 124, no. 5, pp. 570–577, 2016.

[33] C. B. Breckenridge, C. Berry, E. T. Chang, R. L. Sielken, and J. S. Mandel, "Association between Parkinson's disease and cigarette smoking, rural living, well-water consumption, farming and pesticide use: Systematic review and meta-analysis," *PLoS One*, vol. 11, no. 4, 2016.

[34] P. M. Carvey, A. Punati, and M. B. Newman, "Progressive dopamine neuron loss in Parkinson's disease: The multiple hit hypothesis," *Cell Transplantation*, vol. 15, no. 3. pp. 239–250, 2006.

[35] J. W. Langston, P. Ballard, J. W. Tetrud, and I. Irwin, "Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis.," *Science*, vol. 219, no. 4587, pp. 979–980, Feb. 1983.

[36] J. William Langston, L. S. Forno, C. S. Rebert, and I. Irwin, “Selective nigral toxicity after systemic administration of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyrine (MPTP) in the squirrel monkey,” *Brain Res.*, vol. 292, no. 2, pp. 390–394, 1984.

[37] J. Blesa and S. Przedborski, “Parkinson’s disease: animal models and dopaminergic cell vulnerability,” *Front. Neuroanat.*, vol. 8, no. December, p. 155, 2014.

[38] W. Dauer and S. Przedborski, “Parkinson’s disease: Mechanisms and models,” *Neuron*, vol. 39, no. 6. pp. 889–909, 2003.

[39] J. Blesa, I. Trigo-Damas, A. Quiroga-Varela, and N. L. del Rey, “Animal Models of Parkinson’s Disease,” in *Challenges in Parkinson’s Disease*, J. Dorszewska and W. Kozubski, Eds. Rijeka: InTech, 2016.

[40] J. Bove, D. Prou, C. Perier, and S. Przedborski, “Toxin-Induced Models of Parkinson’s Disease,” *J. Am. Soc. Experimenal Neurother.*, vol. 2, no. July, pp. 484–494, 2005.

[41] J. Wills, J. Credle, A. W. Oaks, V. Duka, J.-H. H. Lee, J. Jones, and A. Sidhu, “Paraquat, but not maneb, induces synucleinopathy and tauopathy in striata of mice through inhibition of proteasomal and autophagic pathways,” *PLoS One*, vol. 7, no. 1, p. e30745, Jan. 2012.

[42] F. Cicchetti, N. Lapointe, A. Roberge-Tremblay, M. Saint-Pierre, L. Jimenez, B. W. Ficke, and R. E. Gross, “Systemic exposure to paraquat and maneb models early Parkinson’s disease in young adult rats.,” *Neurobiol. Dis.*, vol. 20, no. 2, pp. 360–71, Nov. 2005.

[43] Y. Jiao, L. Lu, R. W. Williams, and R. J. Smeyne, “Genetic dissection of strain dependent paraquat-induced neurodegeneration in the substantia nigra pars compacta.,” *PLoS One*, vol. 7, no. 1, p. e29447, Jan. 2012.

[44] H. H. Liou, R. C. Chen, T. H. Chen, Y. F. Tsai, and M. C. Tsai, “Attenuation of paraquat-induced dopaminergic toxicity on the substantia nigra by (-)-deprenyl in vivo.,” *Toxicol. Appl. Pharmacol.*, vol. 172, no. 1, pp. 37–43, Apr. 2001.

[45] E. N. Mangano, S. Peters, D. Litteljohn, R. So, C. Bethune, J. Bobyn, M. Clarke, and S. Hayley, “Granulocyte macrophage-colony stimulating factor protects against

substantia nigra dopaminergic cell loss in an environmental toxin model of Parkinson's disease," *Neurobiol. Dis.*, vol. 43, no. 1, pp. 99–112, Jul. 2011.

[46] E. N. Mangano and S. Hayley, "Inflammatory priming of the substantia nigra influences the impact of later paraquat exposure: Neuroimmune sensitization of neurodegeneration," *Neurobiol. Aging*, vol. 30, no. 9, pp. 1361–78, Sep. 2009.

[47] V. N. Uversky, J. Li, and A. L. Fink, "Pesticides directly accelerate the rate of alpha-synuclein fibril formation: A possible factor in Parkinson's disease," *FEBS Lett.*, vol. 500, pp. 105–108, 2001.

[48] F. Zhao, W. Wang, C. Wang, S. L. Siedlak, H. Fujioka, B. Tang, and X. Zhu, "Mfn2 Protects Dopaminergic Neurons Exposed to Paraquat Both in vitro and in vivo: Implications for Idiopathic Parkinson's Disease," *Biochim. Biophys. Acta - Mol. Basis Dis.*, p. , 2017.

[49] X.-F. Wu, M. L. Block, W. Zhang, L. Qin, B. Wilson, W.-Q. Zhang, B. Veronesi, and J.-S. Hong, "The role of microglia in paraquat-induced dopaminergic neurotoxicity.," *Antioxid. Redox Signal.*, vol. 7, no. 5–6, pp. 654–661, 2005.

[50] Q. Wang, Y. Liu, and J. Zhou, "Neuroinflammation in Parkinson's disease and its potential as therapeutic target," *Translational Neurodegeneration*. 2015.

[51] P. M. Rappold, M. Cui, A. S. Chesser, J. Tibbett, J. C. Grima, L. Duan, N. Sen, J. A. Javitch, and K. Tieu, "Paraquat neurotoxicity is mediated by the dopamine transporter and organic cation transporter-3.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 108, no. 51, pp. 20766–71, 2011.

[52] Y. J. Jang, J. H. Won, M. J. Back, Z. Fu, J. M. Jang, H. C. Ha, S. B. Hong, M. Chang, and D. K. Kim, "Paraquat induces apoptosis through a mitochondria-dependent pathway in RAW264.7 cells," *Biomol. Ther.*, 2015.

[53] K. Shimizu, K. Ohtaki, K. Matsubara, K. Aoyama, T. Uezono, O. Saito, M. Suno, K. Ogawa, N. Hayase, K. Kimura, and H. Shiono, "Carrier-mediated processes in blood-brain barrier penetration and neural uptake of paraquat," *Brain Res.*, vol. 906, no. 1–2, pp. 135–142, 2001.

[54] A. L. McCormack and D. A. Di Monte, “Effects of L-dopa and other amino acids against paraquat-induced nigrostriatal degeneration,” *J. Neurochem.*, 2003.

[55] J. Peng, L. L. Peng, F. F. Stevenson, S. R. Doctrow, and J. K. Andersen, “Iron and Paraquat as Synergistic Environmental Risk Factors in Sporadic Parkinson’s Disease Accelerate Age-Related Neurodegeneration,” *J. Neurosci.*, vol. 27, no. 26, pp. 6914–22, Jun. 2007.

[56] J. S. Bus and J. E. Gibson, “Paraquat: Model for oxidant-initiated toxicity,” *Environ. Health Perspect.*, 1984.

[57] B. J. Day and J. D. Crapo, “A metalloporphyrin superoxide dismutase mimetic protects against paraquat-induced lung injury in vivo,” *Toxicol. Appl. Pharmacol.*, 1996.

[58] M. Patel, B. J. Day, J. D. Crapo, I. Fridovich, and J. O. McNamara, “Requirement for superoxide in excitotoxic cell death,” *Neuron*, 1996.

[59] D. A. Drechsel and M. Patel, “Role of reactive oxygen species in the neurotoxicity of environmental agents implicated in Parkinson’s disease,” *Free Radic. Biol. Med.*, vol. 44, no. 11, pp. 1873–1886, Jun. 2008.

[60] P. R. Castello, D. A. Drechsel, and M. Patel, “Mitochondria are a major source of paraquat-induced reactive oxygen species production in the brain,” *J. Biol. Chem.*, vol. 282, no. 19, pp. 14186–14193, May 2007.

[61] S. J. Chinta, A. Rane, K. S. Poksay, D. E. Bredesen, J. K. Andersen, and R. V Rao, “Coupling Endoplasmic Reticulum Stress to the Cell Death Program in Dopaminergic Cells: Effect of Paraquat,” *Neuromolecular Med.*, vol. 10, no. 4, p. 333, Sep. 2008.

[62] D. Bonneh-Barkay, S. H. Reaney, W. J. Langston, and D. A. Di Monte, “Redox cycling of the herbicide paraquat in microglial cultures.,” *Brain Res. Mol. Brain Res.*, vol. 134, no. 1, pp. 52–56, Mar. 2005.

[63] C.-L. Huang, Y.-C. Lee, Y.-C. Yang, T.-Y. Kuo, and N.-K. Huang, “Minocycline prevents paraquat-induced cell death through attenuating endoplasmic reticulum stress and mitochondrial dysfunction.,” *Toxicol. Lett.*, vol. 209, no. 3, pp. 203–210, Mar. 2012.

[64] H. M. Cocheme and M. P. Murphy, “Complex I is the major site of mitochondrial superoxide production by paraquat.,” *J. Biol. Chem.*, vol. 283, no. 4, pp. 1786–1798, Jan. 2008.

[65] A. B. Manning-Bog, A. L. McCormack, J. Li, V. N. Uversky, A. L. Fink, and D. A. Di Monte, “The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: Paraquat and alpha-synuclein,” *J. Biol. Chem.*, vol. 277, no. 3, pp. 1641–1644, Jan. 2002.

[66] A. Zaidi, D. Fernandes, J. L. Bean, and M. L. Michaelis, “Effects of Paraquat-induced Oxidative Stress on the Neuronal Plasma Membrane Ca(2+)-ATPase,” *Free radical biology & medicine*, vol. 47, no. 10. pp. 1507–1514, Nov-2009.

[67] Y. Yoshimura, Y. Watanabe, and T. Shibuya, “Inhibitory effects of calcium channel antagonists on motor dysfunction induced by intracerebroventricular administration of paraquat,” *Pharmacol Toxicol*, 1993.

[68] T. Tawara, T. Fukushima, N. Hojo, A. Isobe, K. Shiwaku, T. Setogawa, and Y. Yamane, “Effects of paraquat on mitochondrial electron transport system and catecholamine contents in rat brain,” *Arch. Toxicol.*, 1996.

[69] C.-L. Huang, C.-C. Chao, Y.-C. Lee, M.-K. Lu, J.-J. Cheng, Y.-C. Yang, V.-C. Wang, W.-C. Chang, and N.-K. Huang, “Paraquat Induces Cell Death Through Impairing Mitochondrial Membrane Permeability.,” *Mol. Neurobiol.*, vol. 53, no. 4, pp. 2169–2188, May 2016.

[70] S. N. P. Kelada, H. Checkoway, S. L. R. Kardia, C. S. Carlson, P. Costa-Mallen, D. L. Eaton, J. Firestone, K. M. Powers, P. D. Swanson, G. M. Franklin, W. T. Longstreth, T.-S. Weller, Z. Afsharinejad, and L. G. Costa, “5’ and 3’ region variability in the dopamine transporter gene (SLC6A3), pesticide exposure and Parkinson’s disease risk: a hypothesis-generating study.,” *Hum. Mol. Genet.*, vol. 15, no. 20, pp. 3055–62, Oct. 2006.

[71] S. Narayan, J. S. Sinsheimer, K. C. Paul, Z. Liew, M. Cockburn, J. M. Bronstein, and B. Ritz, “Genetic variability in ABCB1, occupational pesticide exposure, and

Parkinson's disease," *Environ. Res.*, vol. 143, pp. 98–106, 2015.

[72] M. Westerlund, A. C. Belin, A. Anvret, A. Håkansson, H. Nissbrandt, C. Lind, O. Sydow, L. Olson, and D. Galter, "Association of a polymorphism in the ABCB1 gene with Parkinson's disease," *Parkinsonism Relat. Disord.*, vol. 15, no. 6, pp. 422–424, 2009.

[73] S. M. Goldman, F. Kamel, G. W. Ross, G. S. Bhudhikanok, J. A. Hoppin, M. Korell, C. Marras, C. Meng, D. M. Umbach, M. Kasten, A. R. Chade, K. Comyns, M. B. Richards, D. P. Sandler, A. Blair, J. W. Langston, and C. M. Tanner, "Genetic modification of the association of paraquat and Parkinson's disease.," *Mov. Disord.*, vol. 27, no. 13, pp. 1652–8, Nov. 2012.

[74] E. M. van de Giessen, M. M. L. de Win, M. W. T. Tanck, W. van den Brink, F. Baas, and J. Booij, "Striatal dopamine transporter availability associated with polymorphisms in the dopamine transporter gene SLC6A3.," *J. Nucl. Med.*, vol. 50, no. 1, pp. 45–52, Jan. 2009.

[75] F. Richter, L. Gabby, K. A. McDowell, C. K. Mulligan, K. De La Rosa, P. C. Sioshansi, F. Mortazavi, I. Cely, L. C. Ackerson, L. Tsan, N. P. Murphy, N. T. Maidment, and M.-F. Chesselet, "Effects of decreased dopamine transporter levels on nigrostriatal neurons and paraquat/maneb toxicity in mice," *Neurobiol. Aging*, vol. 51, pp. 54–66, Mar. 2017.

[76] A. M. Hemmerle, J. W. Dickerson, J. P. Herman, and K. B. Seroogy, "Stress exacerbates experimental Parkinson/'s disease," *Mol Psychiatry*, vol. 19, no. 6, pp. 638–640, Jun. 2014.

[77] A. M. Hemmerle, J. P. Herman, and K. B. Seroogy, "Stress, depression and Parkinson's disease.," *Exp. Neurol.*, vol. 233, no. 1, pp. 79–86, Jan. 2012.

[78] E. J. Hermans, M. J. A. G. Henckens, M. Joëls, and G. Fernández, "Dynamic adaptation of large-scale brain networks in response to acute stressors," *Trends Neurosci.*, vol. 37, no. 6, pp. 304–314, Jun. 2014.

[79] H. Anisman, *An Introduction to Stress and Health*. United Kingdom: SAGE

Publications Ltd, 2014.

[80] B. S. McEwen, N. P. Bowles, J. D. Gray, M. N. Hill, R. G. Hunter, I. N. Karatsoreos, and C. Nasca, “Mechanisms of stress in the brain,” *Nat. Neurosci.*, 2015.

[81] M.-C. Audet and H. Anisman, “Interplay between pro-inflammatory cytokines and growth factors in depressive illnesses.,” *Front. Cell. Neurosci.*, vol. 7, no. May, p. 68, 2013.

[82] S. Hayley, “The neuroimmune-neuroplasticity interface and brain pathology,” *Front. Cell. Neurosci.*, vol. 8, p. 419, Dec. 2014.

[83] S. Hayley and D. Litteljohn, “Neuroplasticity and the next wave of antidepressant strategies.,” *Front. Cell. Neurosci.*, vol. 7, no. November, p. 218, 2013.

[84] A. Cattaneo and M. A. Riva, “Stress-induced mechanisms in mental illness: A role for glucocorticoid signalling,” *Journal of Steroid Biochemistry and Molecular Biology*, vol. 160. pp. 169–174, 2016.

[85] D. Litteljohn, E. Mangano, and S. Hayley, “Common Pathways to Neurodegeneration and Co-morbid Depression,” in *Brain Protection in Schizophrenia, Mood and Cognitive Disorders*, S. M. Ritsner, Ed. Dordrecht: Springer Netherlands, 2010, pp. 185–241.

[86] A. Hiller, J. Quinn, and P. Schmidt, “Does Psychological Stress Affect the Progression of Parkinson’s Disease?,” *Neurology*, vol. 88, no. 16 Supplement, Apr. 2017.

[87] J. M. Finlay and M. J. Zigmond, “The effects of stress on central dopaminergic neurons: possible clinical implications.,” *Neurochem. Res.*, vol. 22, no. 11, pp. 1387–1394, 1997.

[88] S. Sugama, K. Sekiyama, T. Kodama, Y. Takamatsu, T. Takenouchi, M. Hashimoto, C. Bruno, and Y. Kakinuma, “Chronic restraint stress triggers dopaminergic and noradrenergic neurodegeneration: Possible role of chronic stress in the onset of Parkinson’s disease.,” *Brain. Behav. Immun.*, vol. 51, pp. 39–46, Jan. 2016.

[89] P. Blandino, C. J. Barnum, and T. Deak, “The involvement of norepinephrine and microglia in hypothalamic and splenic IL-1beta responses to stress,” *J. Neuroimmunol.*,

vol. 173, no. 1–2, pp. 87–95, 2006.

[90] P. Blandino, C. J. Barnum, L. G. Solomon, Y. Larish, B. S. Lankow, and T. Deak, "Gene expression changes in the hypothalamus provide evidence for regionally-selective changes in IL-1 and microglial markers after acute stress," *Brain. Behav. Immun.*, vol. 23, no. 7, pp. 958–968, 2009.

[91] F. R. Walkera, M. Nilsson, and K. Jones, "Acute and Chronic Stress-Induced Disturbances of Microglial Plasticity, Phenotype and Function," *Curr. Drug Targets*, vol. 14, pp. 1262–1276, Oct. 2013.

[92] S. Sugama, M. Fujita, M. Hashimoto, and B. Conti, "Stress induced morphological microglial activation in the rodent brain: involvement of interleukin-18.," *Neuroscience*, vol. 146, no. 3, pp. 1388–1399, May 2007.

[93] A. Niraula, J. F. Sheridan, and J. P. Godbout, "Microglia Priming with Aging and Stress," *Neuropsychopharmacology*, vol. 42, no. 1. American College of Neuropsychopharmacology, pp. 318–333, Jan-2017.

[94] S. Vyas, A. J. Rodrigues, J. M. Silva, F. Tronche, O. F. X. Almeida, N. Sousa, and I. Sotiropoulos, "Chronic Stress and Glucocorticoids: From Neuronal Plasticity to Neurodegeneration.," *Neural Plast.*, vol. 2016, p. 6391686, 2016.

[95] T. C. Theoharides and A. D. Konstantinidou, "Corticotropin-releasing hormone and the blood-brain-barrier," *Front. Biosci. A J. Virtual Libr.*, vol. 12, pp. 1615–1628, Jan. 2007.

[96] M. G. Frank, M. D. Weber, L. R. Watkins, and S. F. Maier, "Stress-induced neuroinflammatory priming: A liability factor in the etiology of psychiatric disorders," *Neurobiol. Stress*, vol. 4, pp. 62–70, Oct. 2016.

[97] V. H. Perry and C. Holmes, "Microglial priming in neurodegenerative disease," *Nature Reviews Neurology*, vol. 10, no. 4. pp. 217–224, 2014.

[98] J. D. Johnson, K. A. O'Connor, T. Deak, M. Stark, L. R. Watkins, and S. F. Maier, "Prior stressor exposure sensitizes LPS-induced cytokine production.," *Brain. Behav. Immun.*, vol. 16, no. 4, pp. 461–476, Aug. 2002.

[99] E. S. Wohleb, M. L. Hanke, A. W. Corona, N. D. Powell, L. M. Stiner, M. T. Bailey, R. J. Nelson, J. P. Godbout, and J. F. Sheridan, "β-Adrenergic Receptor Antagonism Prevents Anxiety-Like Behavior and Microglial Reactivity Induced by Repeated Social Defeat," *J. Neurosci.*, vol. 31, no. 17, pp. 6277–6288, 2011.

[100] L. K. Smith, N. M. Jadavji, K. L. Colwell, S. Katrina Perehudoff, and G. A. Metz, "Stress accelerates neural degeneration and exaggerates motor symptoms in a rat model of Parkinson's disease," *Eur. J. Neurosci.*, vol. 27, no. 8, pp. 2133–2146, 2008.

[101] Y. Glinka, M. Gassen, and M. B. Youdim, "Mechanism of 6-hydroxydopamine neurotoxicity," *J. Neural Transm.*, vol. 50, pp. 55–66, 1997.

[102] U. Janakiraman, T. Manivasagam, A. J. Thenmozhi, M. M. Essa, R. Barathidasan, C. SaravanaBabu, G. J. Guillemin, and M. A. S. Khan, "Influences of Chronic Mild Stress Exposure on Motor, Non-Motor Impairments and Neurochemical Variables in Specific Brain Areas of MPTP/Probenecid Induced Neurotoxicity in Mice.," *PLoS One*, vol. 11, no. 1, p. e0146671, Jan. 2016.

[103] U. Janakiraman, T. Manivasagam, A. Justin Thenmozhi, C. Dhanalakshmi, M. M. Essa, B.-J. Song, and G. J. Guillemin, "Chronic mild stress augments MPTP induced neurotoxicity in a murine model of Parkinson's disease," *Physiol. Behav.*, vol. 173, pp. 132–143, May 2017.

[104] N. H. Rod, Y. Bordelon, A. Thompson, E. Marcotte, and B. Ritz, "Major life events and development of major depression in Parkinson's disease patients," *Eur. J. Neurol.*, vol. 20, no. 4, pp. 663–670, 2013.

[105] L. Pani, A. Porcella, and G. L. Gessa, "The role of stress in the pathophysiology of the dopaminergic system.," *Mol. Psychiatry*, vol. 5, no. 1, pp. 14–21, Jan. 2000.

[106] E. Izzo, P. P. Sanna, and G. F. Koob, "Impairment of dopaminergic system function after chronic treatment with corticotropin-releasing factor," *Pharmacol. Biochem. Behav.*, vol. 81, no. 4, pp. 701–708, 2005.

[107] K. Urakami, N. Masaki, K. Shimoda, S. Nishikawa, and K. Takahashi, "Increase of striatal dopamine turnover by stress in MPTP-treated mice.," *Clin. Neuropharmacol.*,

vol. 11, no. 4, pp. 360–368, Aug. 1988.

[108] A. Kibel and I. Drenjančević-Perić, “Impact of glucocorticoids and chronic stress on progression of Parkinson’s disease,” *Med. Hypotheses*, vol. 71, no. 6, pp. 952–956, 2008.

[109] A. Louveau, T. H. Harris, and J. Kipnis, “Revisiting the Mechanisms of CNS Immune Privilege,” *Trends in Immunology*. 2015.

[110] L. Du, Y. Zhang, Y. Chen, J. Zhu, Y. Yang, and H.-L. Zhang, “Role of Microglia in Neurological Disorders and Their Potentials as a Therapeutic Target.,” *Mol. Neurobiol.*, Nov. 2016.

[111] N. A. Punchard, C. J. Whelan, and I. Adcock, “The Journal of Inflammation,” *Journal of Inflammation*, vol. 1. 2004.

[112] C. J. Janeway, P. Travers, and M. Walport, “Immunobiology: The Immune System in Health and Disease. 5th edition.,” *Garl. Sci.*, 2001.

[113] C. a Janeway and R. Medzhitov, “Innate immune recognition.,” *Annu. Rev. Immunol.*, vol. 20, no. 2, pp. 197–216, 2002.

[114] R. Shtrichman and C. E. Samuel, “The role of gamma interferon in antimicrobial immunity,” *Current Opinion in Microbiology*, vol. 4, no. 3. pp. 251–259, 2001.

[115] H. A. Jurgens and R. W. Johnson, “Dysregulated neuronal-microglial cross-talk during aging, stress and inflammation.,” *Exp. Neurol.*, vol. 233, no. 1, pp. 40–8, Jan. 2012.

[116] U. Saeed, J. Compagnone, R. I. Aviv, A. P. Strafella, S. E. Black, A. E. Lang, and M. Masellis, “Imaging biomarkers in Parkinson’s disease and Parkinsonian syndromes: current and emerging concepts.,” *Transl. Neurodegener.*, vol. 6, p. 8, 2017.

[117] H. Neumann, M. R. Kotter, and R. J. M. Franklin, “Debris clearance by microglia: An essential link between degeneration and regeneration,” *Brain*, vol. 132, no. 2. pp. 288–295, 2009.

[118] A. R. Simard, D. Soulet, G. Gowing, J. P. Julien, and S. Rivest, “Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer’s disease,” *Neuron*, vol. 49, no. 4, pp. 489–502, 2006.

[119] C. Béchade, Y. Cantaut-Belarif, and A. Bessis, “Microglial control of neuronal activity,” *Front. Cell. Neurosci.*, vol. 7, 2013.

[120] C. Gomes, R. Ferreira, J. George, R. Sanches, D. I. Rodrigues, N. Gonçalves, and R. A. Cunha, “Activation of microglial cells triggers a release of brain-derived neurotrophic factor (BDNF) inducing their proliferation in an adenosine A2A receptor-dependent manner: A2A receptor blockade prevents BDNF release and proliferation of microglia,” *J. Neuroinflammation*, vol. 10, 2013.

[121] K. A. Kigerl, J. P. de Rivero Vaccari, W. D. Dietrich, P. G. Popovich, and R. W. Keane, “Pattern recognition receptors and central nervous system repair.,” *Exp. Neurol.*, vol. 258, pp. 5–16, Aug. 2014.

[122] W. Le, J. Wu, and Y. Tang, “Protective Microglia and Their Regulation in Parkinson’s Disease,” *Front. Mol. Neurosci.*, vol. 9, 2016.

[123] L. Tian, L. Ma, T. Kaarela, and Z. Li, “Neuroimmune crosstalk in the central nervous system and its significance for neurological diseases,” *Journal of Neuroinflammation*, vol. 9. 2012.

[124] H. S. Park, M. J. Park, and M. S. Kwon, “Central nervous system-peripheral immune system dialogue in neurological disorders: Possible application of neuroimmunology in urology,” *International Neurourology Journal*, vol. 20. pp. S8–S14, 2016.

[125] S. Hayley, “Toward an Anti-Inflammatory Strategy for Depression,” *Front. Behav. Neurosci.*, vol. 5, 2011.

[126] G. M. Hayes, M. N. Woodroofe, and M. L. Cuzner, “Microglia are the major cell type expressing MHC class II in human white matter,” *J. Neurol. Sci.*, vol. 80, no. 1, pp. 25–37, 1987.

[127] M. Strachan-Whaley, S. Rivest, and V. W. Yong, “Interactions Between Microglia and T Cells in Multiple Sclerosis Pathobiology,” *J. Interf. Cytokine Res.*, vol. 34, no. 8, pp. 615–622, 2014.

[128] M. È. Tremblay and A. Sierra, *Microglia in health and disease*. 2014.

[129] T. Wang, L. Qin, B. Liu, Y. Liu, B. Wilson, T. E. Eling, R. Langenbach, S. Taniura,

and J.-S. Hong, “Role of reactive oxygen species in LPS-induced production of prostaglandin E2 in microglia,” *J. Neurochem.*, vol. 88, no. 4, pp. 939–947, 2004.

[130] T. B. Bassani, M. A. B. F. Vital, and L. K. Rauh, “Neuroinflammation in the pathophysiology of Parkinson’s disease and therapeutic evidence of anti-inflammatory drugs.,” *Arq. Neuropsiquiatr.*, vol. 73, no. 7, pp. 616–23, 2015.

[131] M. Molteni and C. Rossetti, “Neurodegenerative diseases: The immunological perspective,” *Journal of Neuroimmunology*, vol. 313. pp. 109–115, 2017.

[132] W. A. Banks, “Blood-brain barrier transport of cytokines: a mechanism for neuropathology.,” *Curr. Pharm. Des.*, vol. 11, no. 8, pp. 973–84, 2005.

[133] C. T. Capaldo, A. E. Farkas, R. S. Hilgarth, S. M. Krug, M. F. Wolf, J. K. Benedik, M. Fromm, M. Koval, C. Parkos, and A. Nusrat, “Proinflammatory cytokine-induced tight junction remodeling through dynamic self-assembly of claudins,” *Molecular Biology of the Cell*, vol. 25, no. 18. pp. 2710–2719, Sep-2014.

[134] M. Russo and D. B. McGavern, “Immune surveillance of the CNS following infection and injury,” *Trends Immunol.*, vol. 36, no. 10, pp. 637–650, Oct. 2015.

[135] B. P. Daniels, D. W. Holman, L. Cruz-Orengo, H. Jujjavarapu, D. M. Durrant, and R. S. Klein, “Viral pathogen-associated molecular patterns regulate blood-brain barrier integrity via competing innate cytokine signals.,” *MBio*, vol. 5, no. 5, pp. e01476-14, Aug. 2014.

[136] A. Varatharaj and I. Galea, “The blood-brain barrier in systemic inflammation,” *Brain. Behav. Immun.*, vol. 60, pp. 1–12, Feb. 2017.

[137] D. C. German, T. Eagar, and P. K. Sonsalla, “Parkinson’s Disease: A Role for the Immune System,” *Curr. Mol. Pharmacol.*, Jun. 2011.

[138] M. Sawada, K. Imamura, and T. Nagatsu, “Role of cytokines in inflammatory process in Parkinson’s disease.,” *J. Neural Transm. Suppl.*, no. 70, pp. 373–381, 2006.

[139] P. L. McGeer and E. G. McGeer, “Inflammation and neurodegeneration in Parkinson’s disease.,” *Parkinsonism Relat. Disord.*, vol. 10 Suppl 1, pp. S3-7, May 2004.

[140] C. W. Ip, S. K. Beck, and J. Volkmann, “Lymphocytes reduce nigrostriatal deficits in

the 6-hydroxydopamine mouse model of Parkinson's disease," *J. Neural Transm.*, vol. 122, no. 12, pp. 1633–1643, 2015.

[141] P. Thakur, L. S. Breger, M. Lundblad, O. W. Wan, B. Mattsson, K. C. Luk, V. M. Y. Lee, J. Q. Trojanowski, and A. Björklund, "Modeling Parkinson's disease pathology by combination of fibril seeds and α-synuclein overexpression in the rat brain," *Proc. Natl. Acad. Sci.*, p. 201710442, 2017.

[142] V. Brochard, B. Combadière, A. Prigent, Y. Laouar, A. Perrin, V. Beray-Berthat, O. Bonduelle, D. Alvarez-Fischer, J. Callebert, J.-M. Launay, C. Duyckaerts, R. A. Flavell, E. C. Hirsch, and S. Hunot, "Infiltration of CD4(+) lymphocytes into the brain contributes to neurodegeneration in a mouse model of Parkinson disease ," *J. Clin. Invest.*, vol. 119, no. 1, pp. 182–192, Jan. 2009.

[143] N. Kustrimovic, E. Rasini, M. Legnaro, R. Bombelli, I. Aleksic, F. Blandini, C. Comi, M. Mauri, B. Minafra, G. Riboldazzi, V. Sanchez-Guajardo, F. Marino, and M. Cosentino, "Dopaminergic Receptors on CD4+ T Naive and Memory Lymphocytes Correlate with Motor Impairment in Patients with Parkinson's Disease," *Sci. Rep.*, vol. 6, p. 33738, Sep. 2016.

[144] C. Cebrian, J. D. Loike, and D. Sulzer, "Neuronal MHC-I expression and its implications in synaptic function, axonal regeneration and Parkinson's and other brain diseases," *Front. Neuroanat.*, vol. 8, 2014.

[145] T. Alberio, L. Lopiano, and M. Fasano, "Cellular models to investigate biochemical pathways in Parkinson's disease," *FEBS Journal*, vol. 279, no. 7. pp. 1146–1155, 2012.

[146] H. E. Gendelman and S. H. Appel, "Neuroprotective activities of regulatory T cells," *Trends in Molecular Medicine*, vol. 17, no. 12. pp. 687–688, 2011.

[147] H. González and R. Pacheco, "T-cell-mediated regulation of neuroinflammation involved in neurodegenerative diseases," *Journal of Neuroinflammation*, vol. 11, no. 1. 2014.

[148] N. Dzamko, C. L. Geczy, and G. M. Halliday, "Inflammation is genetically implicated in Parkinson's disease," *Neuroscience*, vol. 302, 2015.

[149] N. P. Rocha, A. S. de Miranda, and A. L. Teixeira, “Insights into Neuroinflammation in Parkinson’s Disease: From Biomarkers to Anti-Inflammatory Based Therapies.,” *Biomed Res. Int.*, vol. 2015, p. 628192, 2015.

[150] D. J. Hare, B. R. Cardoso, E. P. Raven, K. L. Double, D. I. Finkelstein, E. A. Szymlek-Gay, and B.-A. Biggs, “Excessive early-life dietary exposure: a potential source of elevated brain iron and a risk factor for Parkinson’s disease,” *npj Park. Dis.*, vol. 3, no. 1, p. 1, 2017.

[151] B. Liu, H. Chen, F. Fang, A. Tillander, and K. Wirdefeldt, “Early-Life Factors and Risk of Parkinson’s Disease: A Register-Based Cohort Study.,” *PLoS One*, vol. 11, no. 4, p. e0152841, 2016.

[152] Z. Ling, D. A. Gayle, S. Y. Ma, J. W. Lipton, C. W. Tong, J.-S. Hong, and P. M. Carvey, “In utero bacterial endotoxin exposure causes loss of tyrosine hydroxylase neurons in the postnatal rat midbrain.,” *Mov. Disord.*, vol. 17, no. 1, pp. 116–124, Jan. 2002.

[153] T. Arimoto and G. Bing, “Up-regulation of inducible nitric oxide synthase in the substantia nigra by lipopolysaccharide causes microglial activation and neurodegeneration.,” *Neurobiol. Dis.*, vol. 12, no. 1, pp. 35–45, Feb. 2003.

[154] L. Qin, X. Wu, M. L. Block, Y. Liu, G. R. Breese, J.-S. Hong, D. J. Knapp, and F. T. Crews, “Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration.,” *Glia*, vol. 55, no. 5, pp. 453–462, Apr. 2007.

[155] C.-H. Chien, M.-J. Lee, H.-C. Liou, H.-H. Liou, and W.-M. Fu, “Microglia-Derived Cytokines/Chemokines Are Involved in the Enhancement of LPS-Induced Loss of Nigrostriatal Dopaminergic Neurons in DJ-1 Knockout Mice,” *PLoS One*, vol. 11, no. 3, p. e0151569, Mar. 2016.

[156] Y.-C. Lu, W.-C. Yeh, and P. S. Ohashi, “LPS/TLR4 signal transduction pathway.,” *Cytokine*, vol. 42, no. 2, pp. 145–151, May 2008.

[157] R. Gorina, M. Font-Nieves, L. Marquez-Kisinousky, T. Santalucia, and A. M. Planas, “Astrocyte TLR4 activation induces a proinflammatory environment through the

interplay between MyD88-dependent NFkappaB signaling, MAPK, and Jak1/Stat1 pathways.," *Glia*, vol. 59, no. 2, pp. 242–255, Feb. 2011.

[158] C. S. Jack, N. Arbour, J. Manusow, V. Montgrain, M. Blain, E. McCrea, A. Shapiro, and J. P. Antel, "TLR signaling tailors innate immune responses in human microglia and astrocytes.," *J. Immunol.*, vol. 175, no. 7, pp. 4320–4330, Oct. 2005.

[159] J. C. Marshall, "Lipopolysaccharide: An Endotoxin or an Exogenous Hormone?," *Clin. Infect. Dis.*, 2005.

[160] D. Trudler, D. Farfara, and D. Frenkel, "Toll-like receptors expression and signaling in glia cells in neuro-amyloidogenic diseases: towards future therapeutic application.," *Mediators Inflamm.*, vol. 2010, 2010.

[161] J. Drouin-Ouellet, I. St-Amour, M. Saint-Pierre, J. Lamontagne-Proulx, J. Kriz, R. A. Barker, and F. Cicchetti, "Toll-Like Receptor Expression in the Blood and Brain of Patients and a Mouse Model of Parkinson's Disease," *International Journal of Neuropsychopharmacology*, vol. 18, no. 6. US, Apr-2015.

[162] M. A. Panaro, D. D. Lofrumento, C. Saponaro, F. De Nuccio, A. Cianciulli, V. Mitolo, and G. Nicolardi, "Expression of TLR4 and CD14 in the central nervous system (CNS) in a MPTP mouse model of Parkinson's-like disease.," *Immunopharmacol. Immunotoxicol.*, vol. 30, no. 4, pp. 729–40, 2008.

[163] F. Ros-Bernal, S. Hunot, M. T. Herrero, S. Parnadeau, J.-C. J.-C. Corvol, L. Lu, D. Alvarez-Fischer, M. A. Carrillo-de Sauvage, F. Saurini, C. Coussieu, K. Kinugawa, A. Prigent, G. Hoglinger, M. Hamon, F. Tronche, E. C. Hirsch, S. Vyas, G. Höglinger, M. Hamon, F. Tronche, E. C. Hirsch, and S. Vyas, "Microglial glucocorticoid receptors play a pivotal role in regulating dopaminergic neurodegeneration in parkinsonism.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 108, no. 16, pp. 6632–7, Apr. 2011.

[164] J. P. L. Daher, L. A. Volpicelli-Daley, J. P. Blackburn, M. S. Moehle, and A. B. West, "Abrogation of α-synuclein-mediated dopaminergic neurodegeneration in LRRK2-deficient rats," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 111, no. 25, pp. 9289–94, 2014.

[165] S. Tanaka, A. Ishii, H. Ohtaki, S. Shioda, T. Yoshida, and S. Numazawa, "Activation

of microglia induces symptoms of Parkinson's disease in wild-type, but not in IL-1 knockout mice.," *J. Neuroinflammation*, vol. 10, p. 143, Dec. 2013.

[166] W.-G. Kim, R. P. Mohney, B. Wilson, G.-H. Jeohn, B. Liu, and J.-S. Hong, "Regional Difference in Susceptibility to Lipopolysaccharide-Induced Neurotoxicity in the Rat Brain: Role of Microglia," *J. Neurosci.*, vol. 20, no. 16, p. 6309 LP-6316, Aug. 2000.

[167] V. L. Savchenko, J. A. McKanna, I. R. Nikonenko, and G. G. Skibo, "Microglia and astrocytes in the adult rat brain: Comparative immunocytochemical analysis demonstrates the efficacy of lipocortin 1 immunoreactivity," *Neuroscience*, 2000.

[168] J. Bobyn, E. N. Mangano, A. Gandhi, E. Nelson, K. Moloney, M. Clarke, and S. Hayley, "Viral-toxin interactions and Parkinson's disease: poly I:C priming enhanced the neurodegenerative effects of paraquat.," *J. Neuroinflammation*, vol. 9, no. 1, p. 86, Jan. 2012.

[169] V. Machado, T. Zöller, A. Attaai, and B. Spittau, "Microglia-mediated neuroinflammation and neurotrophic factor-induced protection in the MPTP mouse model of parkinson's disease-lessons from transgenic mice," *International Journal of Molecular Sciences*, vol. 17, no. 2. 2016.

[170] S. L. Byler, G. W. Boehm, J. D. Karp, R. A. Kohman, A. J. Tarr, T. Schallert, and T. M. Barth, "Systemic lipopolysaccharide plus MPTP as a model of dopamine loss and gait instability in C57Bl/6J mice.," *Behav. Brain Res.*, vol. 198, no. 2, pp. 434–439, Mar. 2009.

[171] M. G. Purisai, A. L. McCormack, S. Cumine, J. Li, M. Z. Isla, and D. A. Di Monte, "Microglial activation as a priming event leading to paraquat-induced dopaminergic cell degeneration," *Neurobiol. Dis.*, vol. 25, no. 2, pp. 392–400, Feb. 2007.

[172] D. N. Doll, E. B. Engler-Chiurazzi, S. E. Lewis, H. Hu, A. E. Kerr, X. Ren, and J. W. Simpkins, "Lipopolysaccharide exacerbates infarct size and results in worsened post-stroke behavioral outcomes," *Behav. Brain Funct.*, vol. 11, no. 1, 2015.

[173] B. Marsh, S. L. Stevens, A. E. B. Packard, B. Gopalan, B. Hunter, P. Y. Leung, C. A. Harrington, and M. P. Stenzel-Poore, "Systemic LPS protects the brain from ischemic

injury by reprogramming the brain's response to stroke: a critical role for IRF3," *J. Neurosci.*, vol. 29, no. 31, pp. 9839–9849, 2010.

[174] F. Aloisi, "Immune function of microglia," *Glia*, vol. 36, no. 2, pp. 165–179, 2001.

[175] K. Ozaki and W. J. Leonard, "Cytokine and cytokine receptor pleiotropy and redundancy," *Journal of Biological Chemistry*, vol. 277, no. 33. pp. 29355–29358, 2002.

[176] J. L. Tchelingerian, J. Quinonero, J. Booss, and C. Jacque, "Localization of TNFα and IL-1a immunoreactivities in striatal neurons after surgical injury to the hippocampus," *Neuron*, vol. 10, no. 2, pp. 213–224, 1993.

[177] G. E. Ringheim, K. L. Burgher, and J. A. Heroux, "Interleukin-6 mRNA expression by cortical neurons in culture: Evidence for neuronal sources of interleukin-6 production in the brain," *J. Neuroimmunol.*, vol. 63, no. 2, pp. 113–123, 1995.

[178] E. N. Benveniste, "Cytokine actions in the central nervous system," *Cytokine and Growth Factor Reviews*, vol. 9, no. 3–4. pp. 259–275, 1998.

[179] A. Vezzani and B. Viviani, "Neuromodulatory properties of inflammatory cytokines and their impact on neuronal excitability," *Neuropharmacology*, 2015.

[180] K. D. Rochfort and P. M. Cummins, "The blood-brain barrier endothelium: a target for pro-inflammatory cytokines.," *Biochem. Soc. Trans.*, vol. 43, no. 4, pp. 702–706, Aug. 2015.

[181] M. M. A. Almutairi, C. Gong, Y. G. Xu, Y. Chang, and H. Shi, "Factors controlling permeability of the blood-brain barrier.," *Cell. Mol. Life Sci.*, vol. 73, no. 1, pp. 57–77, Jan. 2016.

[182] M. D. Turner, B. Nedjai, T. Hurst, and D. J. Pennington, "Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease," *Biochimica et Biophysica Acta - Molecular Cell Research*, vol. 1843, no. 11. pp. 2563–2582, 2014.

[183] T. Lawrence, "The nuclear factor NF-kappaB pathway in inflammation.," *Cold Spring Harbor perspectives in biology*, vol. 1, no. 6. 2009.

[184] T. Hanada and A. Yoshimura, "Regulation of cytokine signaling and inflammation,"

Cytokine Growth Factor Rev., vol. 13, no. 4, pp. 413–421, 2002.

[185] R. J. Davis, "Signal Transduction by the JNK Group of MAP Kinases," *Cell*, vol. 103, no. 2, pp. 239–252, 2000.

[186] J. Liu and A. Lin, "Role of JNK activation in apoptosis: a double-edged sword.," *Cell Res.*, vol. 15, no. 1, pp. 36–42, 2005.

[187] D. Litteljohn, E. N. Mangano, and S. Hayley, "Cyclooxygenase-2 deficiency modifies the neurochemical effects, motor impairment and co-morbid anxiety provoked by paraquat administration in mice.," *Eur. J. Neurosci.*, vol. 28, no. 4, pp. 707–16, Aug. 2008.

[188] G. Ramesh, A. G. Maclean, and M. T. Philipp, "Cytokines and chemokines at the crossroads of neuroinflammation, neurodegeneration, and neuropathic pain," *Mediators Inflamm.*, vol. 2013, 2013.

[189] J. E. Jung, G. S. Kim, and P. H. Chan, "Neuroprotection by interleukin-6 is mediated by signal transducer and activator of transcription 3 and antioxidative signaling in ischemic stroke," *Stroke*, vol. 42, no. 12, pp. 3574–3579, 2011.

[190] K. W. Hofmann, A. F. S. Schuh, J. Saute, R. Townsend, D. Fricke, R. Leke, D. O. Souza, L. V. Portela, M. L. F. Chaves, and C. R. M. Rieder, "Interleukin-6 serum levels in patients with parkinson's disease," *Neurochem. Res.*, vol. 34, no. 8, pp. 1401–1404, 2009.

[191] K. Krei, S. Fredrikson, A. Fontana, and H. Link, "Interleukin-6 is elevated in plasma in multiple sclerosis," *J. Neuroimmunol.*, vol. 31, no. 2, pp. 147–153, 1991.

[192] J. Kálmán, A. Juhász, G. Laird, P. Dickens, T. Járdánházy, A. Rimanóczy, I. Boncz, W. L. I. Parry-Jones, and Z. Janka, "Serum interleukin-6 levels correlate with the severity of dementia in Down syndrome and in Alzheimer's disease," *Acta Neurol. Scand.*, vol. 96, no. 4, pp. 236–240, 2009.

[193] C. Bate, S. Kempster, V. Last, and A. Williams, "Interferon-gamma increases neuronal death in response to amyloid-beta1-42.," *J. Neuroinflammation*, vol. 3, p. 7, 2006.

[194] M. K. McCoy and M. G. Tansey, "TNF signaling inhibition in the CNS: Implications

for normal brain function and neurodegenerative disease," *Journal of Neuroinflammation*, vol. 5. 2008.

[195] A. Maczurek, K. Hager, M. Kenklies, M. Sharman, R. Martins, J. Engel, D. A. Carlson, and G. Münch, "Lipoic acid as an anti-inflammatory and neuroprotective treatment for Alzheimer's disease," *Advanced Drug Delivery Reviews*, vol. 60, no. 13–14. pp. 1463–1470, 2008.

[196] A. Ghosh, A. Kanthasamy, J. Joseph, V. Anantharam, P. Srivastava, B. P. Dranka, B. Kalyanaraman, and A. G. Kanthasamy, "Anti-inflammatory and neuroprotective effects of an orally active apocynin derivative in pre-clinical models of Parkinson's disease," *J. Neuroinflammation*, vol. 9, 2012.

[197] I. Ahmed, R. Tamouza, M. Delord, R. Krishnamoorthy, C. Tzourio, C. Mulot, M. Nacfer, J. C. Lambert, P. Beaune, P. Laurent-Puig, M. A. Loriot, D. Charron, and A. Elbaz, "Association between Parkinson's disease and the HLA-DRB1 locus," *Mov. Disord.*, vol. 27, no. 9, pp. 1104–1110, 2012.

[198] X. Liu, R. Cheng, M. Verbitsky, S. Kisselev, A. Browne, H. Mejia-Sanatana, E. D. Louis, L. J. Cote, H. Andrews, C. Waters, B. Ford, S. Frucht, S. Fahn, K. Marder, L. N. Clark, and J. H. Lee, "Genome-wide association study identifies candidate genes for Parkinson's disease in an Ashkenazi Jewish population.," *BMC Med. Genet.*, vol. 12, no. 1, p. 104, 2011.

[199] L. Pihlstrøm, G. Axelsson, K. A. Bjørnarå, N. Dizdar, C. Fardell, L. Forsgren, B. Holmberg, J. P. Larsen, J. Linder, H. Nissbrandt, O.-B. Tysnes, E. Öhman, E. Dietrichs, and M. Toft, "Supportive evidence for 11 loci from genome-wide association studies in Parkinson's disease," *Neurobiol. Aging*, vol. 34, no. 6, p. 1708.e7-1708.e13, 2013.

[200] M. Subramanium, S. Keshavarao, and I. Kim, "Association of TNF-alpha and IL-1beta genes polymorphism in Parkinson's disease," *Parkinsonism Relat. Disord.*, vol. 22, pp. e169–e170, Jan. 2016.

[201] A. D. Wahner, J. S. Sinsheimer, J. M. Bronstein, and B. Ritz, "Inflammatory cytokine

gene polymorphisms and increased risk of Parkinson disease.," *Arch. Neurol.*, vol. 64, no. 6, pp. 836–40, 2007.

[202] H. Cardenas and L. M. Bolin, "Compromised reactive microgliosis in MPTP-lesioned IL-6 KO mice.," *Brain Res.*, vol. 985, no. 1, pp. 89–97, Sep. 2003.

[203] K. Sriram, J. M. Matheson, S. A. Benkovic, D. B. Miller, M. I. Luster, and J. P. O'Callaghan, "Deficiency of TNF receptors suppresses microglial activation and alters the susceptibility of brain regions to MPTP-induced neurotoxicity: role of TNF-alpha.," *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.*, vol. 20, no. 6, pp. 670–682, Apr. 2006.

[204] D. Litteljohn, E. Mangano, N. Shukla, and S. Hayley, "Interferon-gamma deficiency modifies the motor and co-morbid behavioral pathology and neurochemical changes provoked by the pesticide paraquat.," *Neuroscience*, vol. 164, no. 4, pp. 1894–1906, Dec. 2009.

[205] C. Barcia, C. M. Ros, V. Annese, A. Gomez, F. Ros-Bernal, D. Aguado-Yera, M. E. Martinez-Pagan, V. de Pablos, E. Fernandez-Villalba, and M. T. Herrero, "IFN-gamma signaling, with the synergistic contribution of TNF-alpha, mediates cell specific microglial and astroglial activation in experimental models of Parkinson's disease," *Cell Death Dis. Dis*, vol. 2, p. e142, 2011.

[206] D. Litteljohn, A. Cummings, A. Brennan, A. Gill, S. Chunduri, H. Anisman, and S. Hayley, "Interferon-gamma deficiency modifies the effects of a chronic stressor in mice: Implications for psychological pathology," *Brain. Behav. Immun.*, vol. 24, no. 3, pp. 462–73, Mar. 2010.

[207] M. L. Block, L. Zecca, and J. S. Hong, "Microglia-mediated neurotoxicity: uncovering the molecular mechanisms," *Nat Rev Neurosci*, vol. 8, no. 1, pp. 57–69, 2007.

[208] L. J. Peterson and P. M. Flood, "Oxidative stress and microglial cells in Parkinson's disease," *Mediators of Inflammation*. 2012.

[209] S. Alboni, L. Gibellini, C. Montanari, C. Benatti, S. Benatti, F. Tascedda, N. Brunello, A. Cossarizza, and C. M. Pariante, "N-acetyl-cysteine prevents toxic oxidative effects

induced by IFN-α in human neurons," *Int. J. Neuropsychopharmacol.*, vol. 16, no. 8, pp. 1849–1865, 2013.

[210] H. D. Venters, R. Dantzer, and K. W. Kelley, "Tumor necrosis factor-alpha induces neuronal death by silencing survival signals generated by the type I insulin-like growth factor receptor.," *Ann. N. Y. Acad. Sci.*, vol. 917, pp. 210–220, 2000.

[211] V. Dias, E. Junn, and M. M. Mouradian, "The role of oxidative stress in parkinson's disease," *Journal of Parkinson's Disease*, vol. 3, no. 4. pp. 461–491, 2013.

[212] J. Blesa, I. Trigo-Damas, A. Quiroga-Varela, and V. R. Jackson-Lewis, "Oxidative stress and Parkinson's disease.," *Front. Neuroanat.*, vol. 9, p. 91, 2015.

[213] A. Hald and J. Lotharius, "Oxidative stress and inflammation in Parkinson's disease: Is there a causal link?," *Experimental Neurology*, vol. 193, no. 2. pp. 279–290, 2005.

[214] D.-C. Wu, P. Teismann, K. Tieu, M. Vila, V. Jackson-Lewis, H. Ischiropoulos, and S. Przedborski, "NADPH oxidase mediates oxidative stress in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 100, no. 10, pp. 6145–50, 2003.

[215] M. S. Hernandes, G. D. R. Santos, C. C. Café-Mendes, L. S. Lima, C. Scavone, C. D. Munhoz, and L. R. G. Britto, "Microglial Cells Are Involved in the Susceptibility of NADPH Oxidase Knockout Mice to 6-Hydroxy-Dopamine-Induced Neurodegeneration," *PLoS One*, vol. 8, no. 9, 2013.

[216] J. Peng, F. F. Stevenson, M. L. Oo, and J. K. Andersen, "Iron-enhanced paraquat-mediated dopaminergic cell death due to increased oxidative stress as a consequence of microglial activation," *Free Radic. Biol. Med.*, vol. 46, no. 2, pp. 312–320, 2009.

[217] C. Venkateshappa, G. Harish, R. B. Mythri, A. Mahadevan, M. M. S. Bharath, and S. K. Shankar, "Increased oxidative damage and decreased antioxidant function in aging human substantia nigra compared to striatum: implications for Parkinson's disease.," *Neurochem. Res.*, vol. 37, no. 2, pp. 358–369, Feb. 2012.

[218] D. A. Patten, M. Germain, M. A. Kelly, and R. S. Slack, "Reactive oxygen species: Stuck in the middle of neurodegeneration," *Journal of Alzheimer's Disease*, vol. 20,

no. SUPPL.2. 2010.

[219] G. Zheng, L. P. Dwoskin, and P. A. Crooks, “Vesicular monoamine transporter 2: role as a novel target for drug development.,” *AAPS J.*, vol. 8, no. 4, pp. E682-92, Nov. 2006.

[220] D. J. Hare and K. L. Double, “Iron and dopamine: a toxic couple,” *Brain*, vol. 139, no. 4, p. 1026 LP-1035, Mar. 2016.

[221] X. Su and H. J. Federoff, “Immune responses in Parkinson’s disease: interplay between central and peripheral immune systems.,” *Biomed Res. Int.*, vol. 2014, p. 275178, 2014.

[222] J. M. Taylor, B. S. Main, and P. J. Crack, “Neuroinflammation and oxidative stress: Co-conspirators in the pathology of Parkinson’s disease,” *Neurochemistry International*, vol. 62, no. 5. pp. 803–819, 2013.

[223] C. K. Glass, K. Saijo, B. Winner, M. C. Marchetto, and F. H. Gage, “Mechanisms Underlying Inflammation in Neurodegeneration,” *Cell*, vol. 140, no. 6, pp. 918–934, 2010.

[224] L. F. Burbulla and R. Krüger, “Converging environmental and genetic pathways in the pathogenesis of Parkinson’s disease,” in *Journal of the Neurological Sciences*, 2011, vol. 306, no. 1–2, pp. 1–8.

[225] M. A. Nalls, N. Pankratz, C. M. Lill, C. B. Do, D. G. Hernandez, M. Saad, A. L. Destefano, E. Kara, J. Bras, M. Sharma, C. Schulte, M. F. Keller, S. Arepalli, C. Letson, C. Edsall, H. Stefansson, X. Liu, H. Pliner, J. H. Lee, R. Cheng, M. A. Ikram, J. P. A. Ioannidis, G. M. Hadjigeorgiou, J. C. Bis, M. Martinez, J. S. Perlmutter, A. Goate, K. Marder, B. Fiske, M. Sutherland, G. Xiromerisiou, R. H. Myers, L. N. Clark, K. Stefansson, J. A. Hardy, P. Heutink, H. Chen, N. W. Wood, H. Houlden, H. Payami, A. Brice, W. K. Scott, T. Gasser, L. Bertram, N. Eriksson, T. Foroud, and A. B. Singleton, “Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson’s disease,” *Nat. Genet.*, vol. 46, no. 9, pp. 989–993, 2014.

[226] D. M. Crabtree and J. Zhang, “Genetically engineered mouse models of Parkinson’s disease,” *Brain Research Bulletin*, vol. 88, no. 1. pp. 13–32, 2012.

[227] E. K. Tan, H. K. Kwok, L. C. Tan, W. T. Zhao, K. M. Prakash, W. L. Au, R. Pavanni, Y. Y. Ng, W. Satake, Y. Zhao, T. Toda, and J. J. Liu, "Analysis of GWAS-linked loci in Parkinson disease reaffirms PARK16 as a susceptibility locus," *Neurology*, vol. 75, no. 6, pp. 508–512, 2010.

[228] O. A. Ross, A. I. Soto-Ortolaza, M. G. Heckman, J. O. Aasly, N. Abahuni, G. Annesi, J. A. Bacon, S. Bardien, M. Bozi, A. Brice, L. Brighina, C. Van Broeckhoven, J. Carr, M. C. Chartier-Harlin, E. Dardiotis, D. W. Dickson, N. N. Diehl, A. Elbaz, C. Ferrarese, A. Ferraris, B. Fiske, J. M. Gibson, R. Gibson, G. M. Hadjigeorgiou, N. Hattori, J. P. A. Ioannidis, B. Jasinska-Myga, B. S. Jeon, Y. J. Kim, C. Klein, R. Kruger, E. Kyratzi, S. Lesage, C. H. Lin, T. Lynch, D. M. Maraganore, G. D. Mellick, E. Mutez, C. Nilsson, G. Opala, S. S. Park, A. Puschmann, A. Quattrone, M. Sharma, P. A. Silburn, Y. H. Sohn, L. Stefanis, V. Tadic, J. Theuns, H. Tomiyama, R. J. Uitti, E. M. Valente, S. van de Loo, D. K. Vassilatis, C. Vilariño-Güell, L. R. White, K. Wirdefeldt, Z. K. Wszolek, R. M. Wu, and M. J. Farrer, "Association of LRRK2 exonic variants with susceptibility to Parkinson's disease: A case-control study," *Lancet Neurol.*, vol. 10, no. 10, pp. 898–908, 2011.

[229] R. Wallings, C. Manzoni, and R. Bandopadhyay, "Cellular processes associated with LRRK2 function and dysfunction," *FEBS Journal*, vol. 282, no. 15. pp. 2806–2826, 2015.

[230] X. Q. Yuan, Y. P. Chen, B. Cao, B. Zhao, Q. Q. Wei, X. Y. Guo, Y. Yang, L. X. Yuan, and H. F. Shang, "An association analysis of the R1628P and G2385R polymorphisms of the LRRK2 gene in multiple system atrophy in a Chinese population," *Park. Relat. Disord.*, vol. 21, no. 2, pp. 147–149, 2015.

[231] S. Lesage and A. Brice, "Parkinson's disease: From monogenic forms to genetic susceptibility factors," *Human Molecular Genetics*, vol. 18, no. R1. 2009.

[232] D. Vilas, B. Segura, H. C. Baggio, C. Pont-Sunyer, Y. Compta, F. Valldeoriola, M. José Martí, M. Quintana, A. Bayés, J. Hernández-Vara, M. Calopa, M. Aguilar, C. Junqué, and E. Tolosa, "Nigral and striatal connectivity alterations in asymptomatic

LRRK2 mutation carriers: A magnetic resonance imaging study," *Mov. Disord.*, vol. 31, no. 12, pp. 1820–1828, 2016.

[233] D. G. Healy, M. Falchi, S. S. O'Sullivan, V. Bonifati, A. Durr, S. Bressman, A. Brice, J. Aasly, C. P. Zabetian, S. Goldwurm, J. J. Ferreira, E. Tolosa, D. M. Kay, C. Klein, D. R. Williams, C. Marras, A. E. Lang, Z. K. Wszolek, J. Berciano, A. H. Schapira, T. Lynch, K. P. Bhatia, T. Gasser, A. J. Lees, and N. W. Wood, "Phenotype, genotype, and worldwide genetic penetrance of LRRK2-associated Parkinson's disease: a case--control study," *Lancet Neurol*, vol. 7, no. 7, pp. 583–590, 2008.

[234] D. Litteljohn, C. Rudyk, Z. Dwyer, K. Farmer, T. Fortin, and S. Hayley, "The impact of murine LRRK2 G2019S transgene overexpression on acute responses to inflammatory challenge," *Brain. Behav. Immun.*, vol. 67, pp. 246–256, 2018.

[235] B. K. Gilsbach and A. Kortholt, "Structural biology of the LRRK2 GTPase and kinase domains: implications for regulation," *Front. Mol. Neurosci.*, 2014.

[236] A. Price, C. Manzoni, M. R. Cookson, and P. A. Lewis, "The LRRK2 signalling system," *Cell Tissue Res.*, 2018.

[237] B. D. Lee, V. L. Dawson, and T. M. Dawson, "Leucine-rich repeat kinase 2 (LRRK2) as a potential therapeutic target in Parkinson's disease," *Trends in Pharmacological Sciences*, vol. 33, no. 7. pp. 365–373, 2012.

[238] A. B. West, D. J. Moore, C. Choi, S. A. Andrabi, X. Li, D. Dikeman, S. Biskup, Z. Zhang, K.-L. Lim, V. L. Dawson, and T. M. Dawson, "Parkinson's disease-associated mutations in LRRK2 link enhanced GTP-binding and kinase activities to neuronal toxicity.," *Hum. Mol. Genet.*, vol. 16, no. 2, pp. 223–232, Jan. 2007.

[239] A. B. West, D. J. Moore, S. Biskup, A. Bugayenko, W. W. Smith, C. A. Ross, V. L. Dawson, and T. M. Dawson, "Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 102, no. 46, pp. 16842–16847, Nov. 2005.

[240] M. M. Hulihan, L. Ishihara-Paul, J. Kachergus, L. Warren, R. Amouri, R. Elango, R. K. Prinjha, R. Upmanyu, M. Kefi, M. Zouari, S. Ben Sassi, S. Ben Yahmed, G. El

Euch-Fayeche, P. M. Matthews, L. T. Middleton, R. A. Gibson, F. Hentati, and M. J. Farrer, "LRRK2 Gly2019Ser penetrance in Arab-Berber patients from Tunisia: a case-control genetic study," *Lancet Neurol.*, vol. 7, no. 7, pp. 591–594, 2008.

[241] L. J. Ozelius, G. Senthil, R. Saunders-Pullman, E. Ohmann, A. Deligtisch, M. Tagliati, A. L. Hunt, C. Klein, B. Henick, S. M. Hailpern, R. B. Lipton, J. Soto-Valencia, N. Risch, and S. B. Bressman, "LRRK2 G2019S as a Cause of Parkinson's Disease in Ashkenazi Jews," *N. Engl. J. Med.*, vol. 354, no. 4, pp. 424–425, Jan. 2006.

[242] I. Martin, J. W. Kim, V. L. Dawson, and T. M. Dawson, "LRRK2 pathobiology in Parkinson's disease – virtual inclusion," *Journal of Neurochemistry*. pp. 75–76, 2016.

[243] A. Zimprich, S. Biskup, P. Leitner, P. Lichtner, M. Farrer, S. Lincoln, J. Kachergus, M. Hulihan, R. J. Uitti, D. B. Calne, A. J. Stoessl, R. F. Pfeiffer, N. Patenge, I. C. Carbajal, P. Vieregge, F. Asmus, B. Müller-Myhsok, D. W. Dickson, T. Meitinger, T. M. Strom, Z. K. Wszolek, and T. Gasser, "Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology," *Neuron*, vol. 44, no. 4, pp. 601–607, 2004.

[244] M. R. Cookson, "LRRK2 Pathways Leading to Neurodegeneration," *Current Neurology and Neuroscience Reports*. 2015.

[245] I. Martin, J. W. Kim, V. L. Dawson, and T. M. Dawson, "LRRK2 pathobiology in Parkinson's disease," *Journal of Neurochemistry*. 2014.

[246] A. M. Arranz, L. Delbroek, K. Van Kolen, M. R. Guimarães, W. Mandemakers, G. Daneels, S. Matta, S. Calafate, H. Shaban, P. Baatsen, P.-J. De Bock, K. Gevaert, P. Vanden Berghe, P. Verstreken, B. De Strooper, and D. Moechars, "LRRK2 functions in synaptic vesicle endocytosis through a kinase-dependent mechanism.," *J. Cell Sci.*, no. December, pp. 541–552, 2014.

[247] A. M. A. Schreij, M. Chaineau, W. Ruan, S. Lin, P. A. Barker, E. A. Fon, and P. S. McPherson, "LRRK2 localizes to endosomes and interacts with clathrin-light chains to limit Rac1 activation.," *EMBO Rep.*, vol. 16, no. 1, pp. 79–86, Jan. 2015.

[248] S. J. Orenstein, S.-H. Kuo, I. Tasset, E. Arias, H. Koga, I. Fernandez-Carasa, E. Cortes, L. S. Honig, W. Dauer, A. Consiglio, A. Raya, D. Sulzer, and A. M. Cuervo, "Interplay

of LRRK2 with chaperone-mediated autophagy.," *Nat. Neurosci.*, vol. 16, no. 4, pp. 394–406, 2013.

[249] J. Vitte, S. Traver, A. Maues De Paula, S. Lesage, G. Rovelli, O. Corti, C. Duyckaerts, and A. Brice, "Leucine-rich repeat kinase 2 is associated with the endoplasmic reticulum in dopaminergic neurons and accumulates in the core of Lewy bodies in Parkinson disease.," *J. Neuropathol. Exp. Neurol.*, vol. 69, no. 9, pp. 959–972, Sep. 2010.

[250] X. Wang, M. H. Yan, H. Fujioka, J. Liu, A. Wilson-delfosse, S. G. Chen, G. Perry, G. Casadesus, and X. Zhu, "LRRK2 regulates mitochondrial dynamics and function through direct interaction with DLP1," *Hum. Mol. Genet.*, vol. 21, no. 9, pp. 1931–1944, 2012.

[251] H. J. Cho, J. Yu, C. Xie, P. Rudrabhatla, X. Chen, J. Wu, L. Parisiadou, G. Liu, L. Sun, B. Ma, J. Ding, Z. Liu, and H. Cai, "Leucine-rich repeat kinase 2 regulates Sec16A at ER exit sites to allow ER-Golgi export," *EMBO J.*, vol. 33, no. 20, pp. 2314–31, 2014.

[252] K. Häbig, S. Gellhaar, B. Heim, V. Djuric, F. Giesert, W. Wurst, C. Walter, T. Hentrich, O. Riess, and M. Bonin, "LRRK2 guides the actin cytoskeleton at growth cones together with ARHGEF7 and Tropomyosin 4," *Biochim. Biophys. Acta - Mol. Basis Dis.*, vol. 1832, no. 12, pp. 2352–2367, 2013.

[253] G. Sanna, M. G. Del Giudice, C. Crosio, and C. Iaccarino, "LRRK2 and vesicle trafficking.," *Biochem. Soc. Trans.*, vol. 40, no. 5, pp. 1117–1122, 2012.

[254] C. C.-Y. Ho, H. J. Rideout, E. Ribe, C. M. Troy, and W. T. Dauer, "The Parkinson disease protein leucine-rich repeat kinase 2 transduces death signals via Fas-associated protein with death domain and caspase-8 in a cellular model of neurodegeneration.," *J. Neurosci.*, vol. 29, no. 4, pp. 1011–6, 2009.

[255] J. Umeno, K. Asano, T. Matsushita, T. Matsumoto, Y. Kiyohara, M. Iida, Y. Nakamura, N. Kamatani, and M. Kubo, "Meta-analysis of published studies identified eight additional common susceptibility loci for Crohn's disease and ulcerative colitis," *Inflamm. Bowel Dis.*, vol. 17, no. 12, pp. 2407–2415, 2011.

[256] F.-R. Zhang, W. Huang, S.-M. Chen, L.-D. Sun, H. Liu, Y. Li, Y. Cui, X.-X. Yan, H.-T. Yang, R.-D. Yang, T.-S. Chu, C. Zhang, L. Zhang, J.-W. Han, G.-Q. Yu, C. Quan, Y.-X. Yu, Z. Zhang, B.-Q. Shi, L.-H. Zhang, H. Cheng, C.-Y. Wang, Y. Lin, H.-F. Zheng, X.-A. Fu, X.-B. Zuo, Q. Wang, H. Long, Y.-P. Sun, Y.-L. Cheng, H.-Q. Tian, F.-S. Zhou, H.-X. Liu, W.-S. Lu, S.-M. He, W.-L. Du, M. Shen, Q.-Y. Jin, Y. Wang, H.-Q. Low, T. Erwin, N.-H. Yang, J.-Y. Li, X. Zhao, Y.-L. Jiao, L.-G. Mao, G. Yin, Z.-X. Jiang, X.-D. Wang, J.-P. Yu, Z.-H. Hu, C.-H. Gong, Y.-Q. Liu, R.-Y. Liu, D.-M. Wang, D. Wei, J.-X. Liu, W.-K. Cao, H.-Z. Cao, Y.-P. Li, W.-G. Yan, S.-Y. Wei, K.-J. Wang, M. L. Hibberd, S. Yang, X.-J. Zhang, and J.-J. Liu, "Genomewide association study of leprosy.," *N. Engl. J. Med.*, vol. 361, no. 27, pp. 2609–18, 2009.

[257] M. S. Moehle, P. J. Webber, T. Tse, N. Sukar, D. G. Standaert, T. M. DeSilva, R. M. Cowell, and A. B. West, "LRRK2 inhibition attenuates microglial inflammatory responses.," *J. Neurosci.*, vol. 32, no. 5, pp. 1602–11, 2012.

[258] J. Miklossy, T. Arai, J.-P. P. Guo, A. Klegeris, S. Yu, E. G. McGeer, and P. L. McGeer, "LRRK2 Expression in Normal and Pathologic Human Brain and in Human Cell Lines," *J. Neuropathol. Exp. Neurol.*, vol. 65, no. 10, pp. 953–963, 2006.

[259] A. Gardet, Y. Benita, C. Li, B. E. Sands, I. Ballester, C. Stevens, J. R. Korzenik, J. D. Rioux, M. J. Daly, R. J. Xavier, and D. K. Podolsky, "LRRK2 Is Involved in the IFN-γ Response and Host Response to Pathogens," *J. Immunol.*, vol. 185, no. 9, pp. 5577–5585, Nov. 2010.

[260] T. Maekawa, M. Kubo, I. Yokoyama, E. Ohta, and F. Obata, "Age-dependent and cell-population-restricted LRRK2 expression in normal mouse spleen," *Biochem. Biophys. Res. Commun.*, vol. 392, no. 3, pp. 431–435, 2010.

[261] M. Hakimi, T. Selvanantham, E. Swinton, R. F. Padmore, Y. Tong, G. Kabbach, K. Venderova, S. E. Girardin, D. E. Bulman, and C. R. Scherzer, "Parkinson's disease-linked LRRK2 is expressed in circulating and tissue immune cells and upregulated following recognition of microbial structures," *J Neural Transm*, vol. 118, 2011.

[262] J. Thévenet, R. Gobert, R. H. van Huijsduijnen, C. Wiessner, and Y. J. Sagot,

"Regulation of LRRK2 expression points to a functional role in human monocyte maturation," *PLoS One*, vol. 6, no. 6, 2011.

[263] J. Schapansky, J. D. Nardozzi, F. Felizia, and M. J. LaVoie, "Membrane recruitment of endogenous LRRK2 precedes its potent regulation of autophagy," *Hum. Mol. Genet.*, vol. 23, no. 16, pp. 4201–4214, 2014.

[264] I. Russo, L. Bubacco, and E. Greggio, "LRRK2 and neuroinflammation: partners in crime in Parkinson's disease?," *Journal of Neuroinflammation*, vol. 11. p. 52, 2014.

[265] I. Russo, G. Berti, N. Plotegher, G. Bernardo, R. Filograna, L. Bubacco, and E. Greggio, "Leucine-rich repeat kinase 2 positively regulates inflammation and down-regulates NF-κB p50 signaling in cultured microglia cells.," *J. Neuroinflammation*, vol. 12, p. 230, 2015.

[266] F. Gillardon, R. Schmid, and H. Draheim, "Parkinson's disease-linked leucine-rich repeat kinase 2(R1441G) mutation increases proinflammatory cytokine release from activated primary microglial cells and resultant neurotoxicity.," *Neuroscience*, vol. 208, pp. 41–48, Apr. 2012.

[267] B. Kim, M. S. Yang, D. Choi, J. H. Kim, H. S. Kim, W. Seol, S. Choi, I. Jou, E. Y. Kim, and E. hye Joe, "Impaired inflammatory responses in murine lrrk2-knockdown brain microglia," *PLoS One*, vol. 7, no. 4, 2012.

[268] N. Dzamko, F. Inesta-Vaquera, J. Zhang, C. Xie, H. Cai, S. Arthur, L. Tan, H. Choi, N. Gray, P. Cohen, P. Pedrioli, K. Clark, and D. R. Alessi, "The IkappaB Kinase Family Phosphorylates the Parkinson's Disease Kinase LRRK2 at Ser935 and Ser910 during Toll-Like Receptor Signaling," *PLoS One*, vol. 7, no. 6, p. e39132, Jun. 2012.

[269] Z. Liu, J. Lee, S. Krummey, W. Lu, H. Cai, and M. J. Lenardo, "The kinase LRRK2 is a regulator of the transcription factor NFAT that modulates the severity of inflammatory bowel disease," *Nat Immunol*, vol. 12, no. 11, pp. 1063–1070, Nov. 2011.

[270] K. S. Kim, P. C. Marcogliese, J. Yang, S. M. Callaghan, V. Resende, E. Abdel-Messih, C. Marras, N. P. Visanji, J. Huang, M. G. Schlossmacher, L. Trinkle-Mulcahy, R. S.

Slack, A. E. Lang, C. L. in I. T. Canadian Lrrk2 in Inflammation Team (CLINT), and D. S. Park, “Regulation of myeloid cell phagocytosis by LRRK2 via WAVE2 complex stabilization is altered in Parkinson’s disease.,” *Proc. Natl. Acad. Sci. U. S. A.*, 2018.

[271] P. C. Marcogliese, S. Abuaish, G. Kabbach, E. Abdel-Messih, S. Seang, G. Li, R. S. Slack, M. E. Haque, K. Venderova, and D. S. Park, “LRRK2(I2020T) functional genetic interactors that modify eye degeneration and dopaminergic cell loss in Drosophila.,” *Hum. Mol. Genet.*, vol. 26, no. 7, pp. 1247–1257, Apr. 2017.

[272] D. F. Marker, J. M. Puccini, T. E. Mockus, J. Barbieri, S.-M. M. Lu, and H. A. Gelbard, “LRRK2 kinase inhibition prevents pathological microglial phagocytosis in response to HIV-1 Tat protein,” *J. Neuroinflammation*, vol. 9, no. 1, p. 261, 2012.

[273] J. M. Puccini, D. F. Marker, T. Fitzgerald, J. Barbieri, C. S. Kim, P. Miller-Rhodes, S.-M. Lu, S. Dewhurst, and H. A. Gelbard, “Leucine-Rich Repeat Kinase 2 Modulates Neuroinflammation and Neurotoxicity in Models of Human Immunodeficiency Virus 1-Associated Neurocognitive Disorders,” *J. Neurosci.*, vol. 35, no. 13, pp. 5271–5283, 2015.

[274] M. S. Moehle, J. P. L. Daher, T. D. Hull, R. Boddu, H. A. Abdelmotilib, J. Mobley, G. T. Kannarkat, M. G. Tansey, and A. B. West, “The G2019S LRRK2 mutation increases myeloid cell chemotactic responses and enhances LRRK2 binding to actin-regulatory proteins,” *Hum. Mol. Genet.*, vol. 24, no. 15, pp. 4250–4267, Aug. 2015.

[275] M. Caesar, S. Zach, C. B. Carlson, K. Brockmann, T. Gasser, and F. Gillardon, “Leucine-rich repeat kinase 2 functionally interacts with microtubules and kinase-dependently modulates cell migration.,” *Neurobiol. Dis.*, vol. 54, pp. 280–288, Jun. 2013.

[276] I. Choi, B. Kim, J.-W. Byun, S. H. Baik, Y. H. Huh, J.-H. Kim, I. Mook-Jung, W. K. Song, J.-H. Shin, H. Seo, Y. H. Suh, I. Jou, S. M. Park, H. C. Kang, and E.-H. Joe, “LRRK2 G2019S mutation attenuates microglial motility by inhibiting focal adhesion kinase,” *Nature Communications*, vol. 6. 2015.

[277] K. McDowell and M. F. Chesselet, “Animal models of the non-motor features of

Parkinson's disease," *Neurobiology of Disease*, vol. 46, no. 3. pp. 597–606, 2012.

[278] O. Kano, K. Ikeda, D. Cridebring, T. Takazawa, Y. Yoshii, and Y. Iwasaki, "Neurobiology of depression and anxiety in Parkinson's disease.," *Parkinsons. Dis.*, vol. 2011, p. 143547, 2011.

[279] Y. Ouchi, E. Yoshikawa, Y. Sekine, M. Futatsubashi, T. Kanno, T. Ogusu, and T. Torizuka, "Microglial activation and dopamine terminal loss in early Parkinson's disease," *Ann. Neurol.*, vol. 57, no. 2, pp. 168–175, 2005.

[280] E. L. Jacob, N. M. Gatto, A. Thompson, Y. Bordelon, and B. Ritz, "Occurrence of depression and anxiety prior to Parkinson's disease," *Park. Relat. Disord.*, vol. 16, no. 9, pp. 576–581, 2010.

[281] L. C. S. Tan, "Mood disorders in Parkinson's disease.," *Parkinsonism Relat. Disord.*, vol. 18 Suppl 1, pp. S74-6, 2012.

[282] H. Yapici Eser, H. A. Bora, and A. Kuruoglu, "Depression and Parkinson disease: prevalence, temporal relationship, and determinants.," *Turkish J. Med. Sci.*, vol. 47, no. 2, pp. 499–503, Apr. 2017.

[283] C. Rudyk, D. Litteljohn, S. Syed, Z. Dwyer, and S. Hayley, "Paraquat and psychological stressor interactions as pertains to Parkinsonian co-morbidity," *Neurobiol. Stress*, vol. 2, pp. 85–93, 2015.

[284] C. A. Rudyk, J. McNeill, N. Prowse, Z. Dwyer, K. Farmer, D. Litteljohn, W. Caldwell, and S. Hayley, "Age and Chronicity of Administration Dramatically Influenced the Impact of Low Dose Paraquat Exposure on Behavior and Hypothalamic-Pituitary-Adrenal Activity ," *Frontiers in Aging Neuroscience* , vol. 9. p. 222, 2017.

[285] D. Litteljohn, E. Nelson, C. Bethune, and S. Hayley, "The effects of paraquat on regional brain neurotransmitter activity, hippocampal BDNF and behavioural function in female mice.," *Neurosci. Lett.*, vol. 502, no. 3, pp. 186–91, Sep. 2011.

[286] A. Wang, S. Costello, M. Cockburn, X. Zhang, J. Bronstein, and B. Ritz, "Parkinson's disease risk from ambient exposure to pesticides," *Eur. J. Epidemiol.*, vol. 26, no. 7, pp. 547–555, Jul. 2011.

[287] C. M. Tanner, G. W. Ross, S. A. Jewell, R. A. Hauser, J. Jankovic, S. A. Factor, S. Bressman, A. Deligtisch, C. Marras, K. E. Lyons, G. S. Bhudhikanok, D. F. Roucoux, C. Meng, R. D. Abbott, and J. W. Langston, "Occupation and risk of parkinsonism: A multicenter case-control study," *Arch. Neurol.*, vol. 66, no. 9, pp. 1106–1113, 2009.

[288] F. Moisan, J. Spinosi, L. Delabre, V. Gourlet, J. L. Mazurie, I. Bénatru, M. Goldberg, M. G. Weisskopf, E. Imbernon, C. Tzourio, and A. Elbaz, "Association of parkinson's disease and its subtypes with agricultural pesticide exposures in men: A case-control study in France," *Environ. Health Perspect.*, vol. 123, no. 11, pp. 1123–1129, 2015.

[289] S. Shrestha, F. Kamel, D. M. Umbach, L. E. Beane Freeman, S. Koutros, M. Alavanja, D. P. Sandler, and H. Chen, "Nonmotor symptoms and Parkinson disease in United States farmers and spouses," *PLoS One*, vol. 12, no. 9, 2017.

[290] S. McCarthy, M. Somayajulu, M. Sikorska, H. Borowy-Borowski, and S. Pandey, "Paraquat induces oxidative stress and neuronal cell death; neuroprotection by water-soluble Coenzyme Q10.," *Toxicol. Appl. Pharmacol.*, vol. 201, no. 1, pp. 21–31, Nov. 2004.

[291] E. N. Mangano, D. Litteljohn, R. So, E. Nelson, S. Peters, C. Bethune, J. Bobyn, and S. Hayley, "Interferon-γ plays a role in paraquat-induced neurodegeneration involving oxidative and proinflammatory pathways," *Neurobiol. Aging*, vol. 33, no. 7, pp. 1411–1426, 2012.

[292] A. Czerniczyniec, A. G. Karadayian, J. Bustamante, R. A. Cutrera, and S. Lores-Arnaiz, "Paraquat induces behavioral changes and cortical and striatal mitochondrial dysfunction," *Free Radic. Biol. Med.*, vol. 51, no. 7, pp. 1428–36, Oct. 2011.

[293] L. Chen, S. E. Yoo, R. Na, Y. Liu, and Q. Ran, "Cognitive impairment and increased Aβ levels induced by paraquat exposure are attenuated by enhanced removal of mitochondrial H 2O 2," *Neurobiol. Aging*, vol. 33, no. 2, 2012.

[294] Y. Ait-Bali, S. Ba-M'hamed, and M. Bennis, "Prenatal Paraquat exposure induces neurobehavioral and cognitive changes in mice offspring," *Environ. Toxicol. Pharmacol.*, vol. 48, pp. 53–62, 2016.

[295] K. Prasad, E. Tarasewicz, J. Mathew, P. A. O. Strickland, B. Buckley, J. R. Richardson, and E. K. Richfield, "Toxicokinetics and toxicodynamics of paraquat accumulation in mouse brain," *Exp. Neurol.*, vol. 215, no. 2, pp. 358–367, 2009.

[296] A. D. Smith, S. L. Castro, and M. J. Zigmond, "Stress-induced Parkinson's disease: a working hypothesis.," *Physiol. Behav.*, vol. 77, no. 4–5, pp. 527–531, Dec. 2002.

[297] K. W. Austin, S. W. Ameringer, and L. J. Cloud, "An Integrated Review of Psychological Stress in Parkinson's Disease: Biological Mechanisms and Symptom and Health Outcomes," *Parkinson's Disease*. 2016.

[298] R. M. De Pablos, A. J. Herrera, A. M. Espinosa-Oliva, M. Sarmiento, M. F. Muñoz, A. Machado, and J. L. Venero, "Chronic stress enhances microglia activation and exacerbates death of nigral dopaminergic neurons under conditions of inflammation," *J. Neuroinflammation*, 2014.

[299] R. M. de Pablos, R. F. Villarán, S. Argüelles, A. J. Herrera, J. L. Venero, A. Ayala, J. Cano, and A. Machado, "Stress Increases Vulnerability to Inflammation in the Rat Prefrontal Cortex," *J. Neurosci.* , 2006.

[300] J. B. Buchanan, N. L. Sparkman, J. Chen, and R. W. Johnson, "Cognitive and neuroinflammatory consequences of mild repeated stress are exacerbated in aged mice," *Psychoneuroendocrinology*, 2008.

[301] M. G. Frank, S. A. Hershman, M. D. Weber, L. R. Watkins, and S. F. Maier, "Chronic exposure to exogenous glucocorticoids primes microglia to pro-inflammatory stimuli and induces NLRP3 mRNA in the hippocampus," *Psychoneuroendocrinology*, 2014.

[302] D. Litteljohn, E. Nelson, and S. Hayley, "IFN-gamma differentially modulates memory-related processes under basal and chronic stressor conditions.," *Front. Cell. Neurosci.*, vol. 8, p. 391, 2014.

[303] D. Litteljohn, A. Cummings, A. Brennan, A. Gill, S. Chunduri, H. Anisman, and S. Hayley, "Interferon-gamma deficiency modifies the effects of a chronic stressor in mice: Implications for psychological pathology," *Brain. Behav. Immun.*, vol. 24, no. 3, pp. 462–473, 2010.

[304] P. Willner, A. Towell, D. Sampson, S. Sophokleous, and R. Muscat, “Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant.,” *Psychopharmacology (Berl).*, vol. 93, no. 3, pp. 358–64, Jan. 1987.

[305] P. M. Wall and C. Messier, “The hippocampal formation--orbitomedial prefrontal cortex circuit in the attentional control of active memory.,” *Behav. Brain Res.*, vol. 127, no. 1–2, pp. 99–117, Dec. 2001.

[306] N. Salmaso, H. E. Stevens, J. McNeill, M. ElSayed, Q. Ren, M. E. Maragnoli, M. L. Schwartz, S. Tomasi, R. M. Sapolsky, R. Duman, and F. M. Vaccarino, “Fibroblast Growth Factor 2 Modulates Hypothalamic Pituitary Axis Activity and Anxiety Behavior Through Glucocorticoid Receptors.,” *Biol. Psychiatry*, vol. 80, no. 6, pp. 479–489, Sep. 2016.

[307] R. D. Porsolt, A. Bertin, and M. Jalfre, “Behavioral despair in mice: a primary screening test for antidepressants.,” *Arch. Int. Pharmacodyn. thérapie*, vol. 229, no. 2, pp. 327–36, Oct. 1977.

[308] K. Ohsawa, Y. Imai, Y. Sasaki, and S. Kohsaka, “Microglia/macrophage-specific protein Iba1 binds to fimbrin and enhances its actin-bundling activity,” *J. Neurochem.*, 2004.

[309] D. Ito, Y. Imai, K. Ohsawa, K. Nakajima, Y. Fukuuchi, and S. Kohsaka, “Microglia-specific localisation of a novel calcium binding protein, Iba1,” *Mol. Brain Res.*, vol. 57, no. 1, pp. 1–9, 1998.

[310] L. Tapia-Arancibia, E. Aliaga, M. Silhol, and S. Arancibia, “New insights into brain BDNF function in normal aging and Alzheimer disease.,” *Brain Res. Rev.*, vol. 59, no. 1, pp. 201–220, Nov. 2008.

[311] W. M. Lambert, C.-F. Xu, T. A. Neubert, M. V Chao, M. J. Garabedian, and F. D. Jeanneteau, “Brain-derived neurotrophic factor signaling rewrites the glucocorticoid transcriptome via glucocorticoid receptor phosphorylation.,” *Mol. Cell. Biol.*, vol. 33, no. 18, pp. 3700–3714, Sep. 2013.

[312] F. L. Campos, M. M. Carvalho, A. C. Cristovão, G. Je, G. Baltazar, A. J. Salgado, Y.-S. Kim, and N. Sousa, "Rodent models of Parkinson's disease: beyond the motor symptomatology," *Front. Behav. Neurosci.*, vol. 7, p. 175, 2013.

[313] L. Yin, L. Lu, K. Prasad, E. K. Richfield, E. L. Unger, J. Xu, and B. C. Jones, "Genetic-based, differential susceptibility to paraquat neurotoxicity in mice.," *Neurotoxicol. Teratol.*, vol. 33, no. 3, pp. 415–21, 2011.

[314] T. Chanyachukul, K. Yoovathaworn, W. Thongsaard, S. Chongthammakun, P. Navasumrit, and J. Satayavivad, "Attenuation of paraquat-induced motor behavior and neurochemical disturbances by L-valine in vivo.," *Toxicol. Lett.*, vol. 150, no. 3, pp. 259–69, May 2004.

[315] S. Mitra, N. Chakrabarti, and A. Bhattacharyya, "Differential regional expression patterns of α-synuclein, TNF-α, and IL-1β; and variable status of dopaminergic neurotoxicity in mouse brain after Paraquat treatment.," *J. Neuroinflammation*, vol. 8, p. 163, Jan. 2011.

[316] Q. Chen, Y. Niu, R. Zhang, H. Guo, Y. Gao, Y. Li, and R. Liu, "The toxic influence of paraquat on hippocampus of mice: Involvement of oxidative stress," *Neurotoxicology*, vol. 31, no. 3, pp. 310–316, 2010.

[317] M. Songin, K. Ossowska, K. Kuter, and J. Strosznajder, "Alteration of GSK-3 β in the hippocampus and other brain structures after chronic paraquat administration in rats," *Folia Neuropathol*, pp. 319–327, 2011.

[318] P. Desplats, P. Patel, K. Kosberg, M. Mante, C. Patrick, E. Rockenstein, M. Fujita, M. Hashimoto, and E. Masliah, "Combined exposure to Maneb and Paraquat alters transcriptional regulation of neurogenesis-related genes in mice models of Parkinson's disease.," *Mol. Neurodegener.*, vol. 7, no. 1, p. 49, Jan. 2012.

[319] P. O. Fernagut, C. B. Hutson, S. M. Fleming, N. A. Tetreaut, J. Salcedo, E. Masliah, and M. F. Chesselet, "Behavioral and histopathological consequences of paraquat intoxication in mice: effects of alpha-synuclein over-expression.," *Synapse*, vol. 61, no. 12, pp. 991–1001, Dec. 2007.

[320] K. Ossowska, M. Śmiałowska, K. Kuter, J. Wierońska, B. Zieba, J. Wardas, P. Nowak, J. Dabrowska, A. Bortel, I. Biedka, G. Schulze, and H. Rommelspacher, "Degeneration of dopaminergic mesocortical neurons and activation of compensatory processes induced by a long-term paraquat administration in rats: Implications for Parkinson's disease," *Neuroscience*, vol. 141, no. 4, pp. 2155–2165, 2006.

[321] H. Anisman, Z. Merali, and S. Hayley, "Neurotransmitter, peptide and cytokine processes in relation to depressive disorder: Comorbidity between depression and neurodegenerative disorders," *Progress in Neurobiology*, vol. 85, no. 1. pp. 1–74, 2008.

[322] S. L. Gourley, D. D. Kiraly, J. L. Howell, P. Olausson, and J. R. Taylor, "Acute Hippocampal Brain-Derived Neurotrophic Factor Restores Motivational and Forced Swim Performance After Corticosterone," *Biol. Psychiatry*, vol. 64, no. 10, pp. 884–890, 2008.

[323] H. D. Schmidt and R. S. Duman, "Peripheral BDNF produces antidepressant-like effects in cellular and behavioral models," *Neuropsychopharmacology*, vol. 35, no. 12, pp. 2378–2391, 2010.

[324] M.-F. F. Marin, C. Lord, J. Andrews, R.-P. P. Juster, S. Sindi, G. Arsenault-Lapierre, A. J. Fiocco, and S. J. Lupien, "Chronic stress, cognitive functioning and mental health," *Neurobiol. Learn. Mem.*, vol. 96, no. 4, pp. 583–595, 2011.

[325] J. P. Herman and W. E. Cullinan, "Neurocircuitry of stress: Central control of the hypothalamo-pituitary-adrenocortical axis," *Trends in Neurosciences*. 1997.

[326] E. Kasahara and M. Inoue, "Cross-talk between HPA-axis-increased glucocorticoids and mitochondrial stress determines immune responses and clinical manifestations of patients with sepsis," *Redox Rep.*, 2015.

[327] Q.-G. Zhou, L.-J. Zhu, C. Chen, H.-Y. Wu, C.-X. Luo, L. Chang, and D.-Y. Zhu, "Hippocampal Neuronal Nitric Oxide Synthase Mediates the Stress-Related Depressive Behaviors of Glucocorticoids by Downregulating Glucocorticoid Receptor," *J. Neurosci.*, 2011.

[328] M. García-Fernández, E. Castilla-Ortega, C. Pedraza, E. Blanco, I. Hurtado-Guerrero, M. A. Barbancho, J. Chun, F. Rodríguez-De-Fonseca, G. Estivill-Torrús, and L. J. S. Núñez, "Chronic immobilization in the malpar1 knockout mice increases oxidative stress in the hippocampus," *Int. J. Neurosci.*, 2012.

[329] M. Moretti, J. Budni, D. B. Dos Santos, A. Antunes, J. F. Daufenbach, L. M. Manosso, M. Farina, and A. L. S. Rodrigues, "Protective effects of ascorbic acid on behavior and oxidative status of restraint-stressed mice," *J. Mol. Neurosci.*, 2013.

[330] M. A. Cenci and M. Lundblad, "Utility of 6-hydroxydopamine lesioned rats in the preclinical screening of novel treatments for parkinson disease," in *Movement Disorders*, 2005.

[331] M. Lundblad, E. Vaudano, and M. A. Cenci, "Cellular and behavioural effects of the adenosine A2a receptor antagonist KW-6002 in a rat model of l-DOPA-induced dyskinesia.," *J. Neurochem.*, 2003.

[332] R. Betarbet, T. B. Sherer, G. MacKenzie, M. Garcia-Osuna, A. V. Panov, and J. T. Greenamyre, "Chronic systemic pesticide exposure reproduces features of Parkinson's disease," *Nat. Neurosci.*, vol. 3, no. 12, pp. 1301–1306, 2000.

[333] C. H. Hawkes, K. Del Tredici, and H. Braak, "Parkinson's disease: A dual-hit hypothesis," *Neuropathology and Applied Neurobiology*, vol. 33, no. 6. pp. 599–614, 2007.

[334] H. A. Boger, A. C. Granholm, J. F. McGinty, and L. D. Middaugh, "A dual-hit animal model for age-related parkinsonism," *Progress in Neurobiology*, vol. 90, no. 2. pp. 217–229, 2010.

[335] C. Freire and S. Koifman, "Pesticide exposure and Parkinson's disease: Epidemiological evidence of association," *NeuroToxicology*, vol. 33, no. 5. pp. 947–971, 2012.

[336] B. R. Ritz, A. D. Manthripragada, S. Costello, S. J. Lincoln, M. J. Farrer, M. Cockburn, and J. Bronstein, "Dopamine transporter genetic variants and pesticides in Parkinson's disease," *Environ. Health Perspect.*, vol. 117, no. 6, pp. 964–969, 2009.

[337] E. Greggio, L. Civiero, M. Bisaglia, and L. Bubacco, “Parkinson’s disease and immune system: Is the culprit LRRKing in the periphery?,” *Journal of Neuroinflammation*, vol. 9. 2012.

[338] R. Chia, S. Haddock, A. Beilina, I. N. Rudenko, A. Mamais, A. Kaganovich, Y. Li, R. Kumaran, M. A. Nalls, and M. R. Cookson, “Phosphorylation of LRRK2 by casein kinase 1α regulates trans-Golgi clustering via differential interaction with ARHGEF7,” *Nat. Commun.*, vol. 5, 2014.

[339] F. Giesert, A. Hofmann, A. Bürger, J. Zerle, K. Kloos, U. Hafen, L. Ernst, J. Zhang, D. M. Vogt-Weisenhorn, and W. Wurst, “Expression Analysis of Lrrk1, Lrrk2 and Lrrk2 Splice Variants in Mice,” *PLoS One*, vol. 8, no. 5, 2013.

[340] W. Mandemakers, A. Snellinx, M. J. O’Neill, and B. de Strooper, “LRRK2 expression is enriched in the striosomal compartment of mouse striatum,” *Neurobiol. Dis.*, vol. 48, no. 3, pp. 582–593, 2012.

[341] D. A. Cory-Slechta, “Studying Toxicants as Single Chemicals: Does this Strategy Adequately Identify Neurotoxic Risk?,” *Neurotoxicology*, vol. 26, no. 4, pp. 491–510, 2005.

[342] C. Cunha, C. Gomes, A. R. Vaz, and D. Brites, “Exploring New Inflammatory Biomarkers and Pathways during LPS-Induced M1 Polarization,” *Mediators Inflamm.*, vol. 2016, 2016.

[343] N. Ibarra, A. Pollitt, and R. H. Insall, “Regulation of actin assembly by SCAR/WAVE proteins,” *Biochem. Soc. Trans.*, vol. 33, no. 6, p. 1243 LP-1246, Oct. 2005.

[344] H. Y. Febinger, H. E. Thomasy, M. N. Pavlova, K. M. Ringgold, P. R. Barf, A. M. George, J. N. Grillo, A. D. Bachstetter, J. A. Garcia, A. E. Cardona, M. R. Opp, and C. Gemma, “Time-dependent effects of CX3CR1 in a mouse model of mild traumatic brain injury,” *J. Neuroinflammation*, vol. 12, no. 1, 2015.

[345] A. E. Cardona, E. P. Pioro, M. E. Sasse, V. Kostenko, S. M. Cardona, I. M. Dijkstra, D. R. Huang, G. Kidd, S. Dombrowski, R. Dutta, J.-C. C. Lee, D. N. Cook, S. Jung, S. A. Lira, D. R. Littman, and R. M. Ransohoff, “Control of microglial neurotoxicity by the

fractalkine receptor," *Nat. Neurosci.*, vol. 9, no. 7, pp. 917–924, 2006.

[346] C. Sara, G. Á. J., L. Tresa, C. Maria, L. J. Luis, and L. Isabel, "Cx3cr1-deficiency exacerbates alpha-synuclein-A53T induced neuroinflammation and neurodegeneration in a mouse model of Parkinson's disease," *Glia*, vol. 0, no. 0, Apr. 2018.

[347] R. H. Insall and L. M. Machesky, "Actin Dynamics at the Leading Edge: From Simple Machinery to Complex Networks," *Developmental Cell*, vol. 17, no. 3. pp. 310–322, 2009.

[348] I. R. Evans, P. A. Ghai, V. Urbančič, K.-L. Tan, and W. Wood, "SCAR/WAVE-mediated processing of engulfed apoptotic corpses is essential for effective macrophage migration in Drosophila," *Cell Death Differ.*, vol. 20, no. 5, pp. 709–720, May 2013.

[349] K. M. Hinkle, M. Yue, B. Behrouz, J. C. Dächsel, S. J. Lincoln, E. E. Bowles, J. E. Beevers, B. Dugger, B. Winner, I. Prots, C. B. Kent, K. Nishioka, W.-L. Lin, D. W. Dickson, C. J. Janus, M. J. Farrer, and H. L. Melrose, "LRRK2 knockout mice have an intact dopaminergic system but display alterations in exploratory and motor co-ordination behaviors.," *Mol. Neurodegener.*, vol. 7, p. 25, 2012.

[350] R. Gordon, C. E. Hogan, M. L. Neal, V. Anantharam, A. G. Kanthasamy, and A. Kanthasamy, "A simple magnetic separation method for high-yield isolation of pure primary microglia.," *J. Neurosci. Methods*, vol. 194, no. 2, pp. 287–296, Jan. 2011.

[351] A. Tinakoua, S. Bouabid, E. Faggiani, P. De Deurwaerdère, N. Lakhdar-Ghazal, and A. Benazzouz, "The impact of combined administration of paraquat and maneb on motor and non-motor functions in the rat," *Neuroscience*, vol. 311, pp. 118–129, 2015.

[352] J. C. Nolz, T. S. Gomez, P. Zhu, S. Li, R. B. Medeiros, Y. Shimizu, J. K. Burkhardt, B. D. Freedman, and D. D. Billadeau, "The WAVE2 complex regulates actin cytoskeletal reorganization and CRAC-mediated calcium entry during T cell activation.," *Curr. Biol.*, vol. 16, no. 1, pp. 24–34, Jan. 2006.

[353] J. Shi, G. Scita, and J. E. Casanova, "WAVE2 signaling mediates invasion of polarized epithelial cells by Salmonella typhimurium," *J. Biol. Chem.*, vol. 280, no. 33, pp.

29849–29855, 2005.

[354] F. B. Patel and M. C. Soto, “WAVE/SCAR promotes endocytosis and early endosome morphology in polarized C. elegans epithelia,” *Dev. Biol.*, vol. 377, no. 2, pp. 319–332, May 2013.

[355] I. Zanoni, C. Bodio, A. Broggi, R. Ostuni, M. Caccia, M. Collini, A. Venkatesh, R. Spreafico, G. Capuano, and F. Granucci, “Similarities and differences of innate immune responses elicited by smooth and rough LPS,” *Immunol. Lett.*, vol. 142, no. 1–2, pp. 41–47, 2012.

[356] S. Hayley, K. Brebner, S. Lacosta, Z. Merali, and H. Anisman, “Sensitization to the effects of tumor necrosis factor-alpha: neuroendocrine, central monoamine, and behavioral variations.,” *J. Neurosci.*, vol. 19, no. 13, pp. 5654–65, 1999.

[357] K. L. Paumier, S. J. Sukoff Rizzo, Z. Berger, Y. Chen, C. Gonzales, E. Kaftan, L. Li, S. Lotarski, M. Monaghan, W. Shen, P. Stolyar, D. Vasilyev, M. Zaleska, W. D Hirst, and J. Dunlop, “Behavioral characterization of A53T mice reveals early and late stage deficits related to Parkinson’s disease.,” *PLoS One*, vol. 8, no. 8, p. e70274, 2013.

[358] K. Nagamoto-Combs and C. K. Combs, “Microglial phenotype is regulated by activity of the transcription factor, NFAT (nuclear factor of activated T cells).,” *J. Neurosci.*, vol. 30, no. 28, pp. 9641–6, 2010.

[359] G. D. Manocha, A. M. Floden, K. L. Puig, K. Nagamoto-Combs, C. R. Scherzer, and C. K. Combs, “Defining the contribution of neuroinflammation to Parkinson’s disease in humanized immune system mice,” *Mol. Neurodegener.*, vol. 12, no. 1, 2017.

[360] F. Longo, D. Mercatelli, S. Novello, L. Arcuri, A. Brugnoli, F. Vincenzi, I. Russo, G. Berti, O. S. Mabrouk, R. T. Kennedy, D. R. Shimshek, K. Varani, L. Bubacco, E. Greggio, and M. Morari, “Age-dependent dopamine transporter dysfunction and Serine129 phospho-α-synuclein overload in G2019S LRRK2 mice,” *Acta Neuropathol. Commun.*, vol. 5, no. 1, p. 22, 2017.

[361] S. S. Karuppagounder, Y. Xiong, Y. Lee, M. C. Lawless, D. Kim, E. Nordquist, I. Martin, P. Ge, S. Brahmachari, A. Jhaldiyal, M. kumar, S. A. Andrabi, T. M. Dawson,

and V. L. Dawson, “LRRK2 G2019S Transgenic Mice Display Increased Susceptibility to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-Mediated Neurotoxicity,” *J. Chem. Neuroanat.*, vol. 76, no. Pt B, pp. 90–97, Oct. 2016.

[362] S. N. Kelada, P. Costa-Mallen, H. Checkoway, C. S. Carlson, T. S. Weller, P. D. Swanson, G. M. Franklin, W. T. Longstreth, Z. Afsharinejad, and L. G. Costa, “Dopamine transporter (SLC6A3) 5 ’ region haplotypes significantly affect transcriptional activity in vitro but are not associated with Parkinson’s disease,” *Pharmacogenet. Genomics*, vol. 15, no. 9, pp. 659–668, 2005.

[363] Y. Tong, H. Yamaguchi, E. Giaime, S. Boyle, R. Kopan, R. J. Kelleher, and J. Shen, “Loss of leucine-rich repeat kinase 2 causes impairment of protein degradation pathways, accumulation of alpha-synuclein, and apoptotic cell death in aged mice.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 107, no. 21, pp. 9879–84, 2010.

[364] P. Gómez-Suaga and S. Hilfiker, “LRRK2 as a modulator of lysosomal calcium homeostasis with downstream effects on autophagy,” *Autophagy*, vol. 8, no. 4. pp. 692–693, 2012.

[365] C. Marras, R. N. Alcalay, C. Caspell-Garcia, C. Coffey, P. Chan, J. E. Duda, M. F. Facheris, R. Fernández-Santiago, J. Ruíz-Martínez, T. Mestre, R. Saunders-Pullman, C. Pont-Sunyer, E. Tolosa, and B. Waro, “Motor and nonmotor heterogeneity of LRRK2-related and idiopathic Parkinson’s disease,” *Mov. Disord.*, vol. 31, no. 8, pp. 1192–1202, 2016.

[366] J. H. Somme, A. Molano Salazar, A. Gonzalez, B. Tijero, K. Berganzo, E. Lezcano, M. Fernandez Martinez, J. J. Zarranz, and J. C. Gómez-Esteban, “Cognitive and behavioral symptoms in Parkinson’s disease patients with the G2019S and R1441G mutations of the LRRK2 gene,” *Park. Relat. Disord.*, vol. 21, no. 5, pp. 494–499, 2015.

[367] A. Barbeau, M. Roy, G. Bernier, G. Campanella, and S. Paris, “Ecogenetics of Parkinson’s disease: prevalence and environmental aspects in rural areas,” *Can J Neurol Sci*, vol. 14, no. 1, pp. 36–41, 1987.

[368] C. Damalas and S. Koutroubas, “Farmers’ Exposure to Pesticides: Toxicity Types and

Ways of Prevention," *Toxics*, vol. 4, no. 1, p. 1, 2016.

[369] K. Prasad, B. Winnik, M. J. Thiruchelvam, B. Buckley, O. Mirochnitchenko, and E. K. Richfield, "Prolonged toxicokinetics and toxicodynamics of paraquat in mouse brain," *Environ. Health Perspect.*, 2007.

[370] A. L. McCormack, M. Thiruchelvam, A. B. Manning-Bog, C. Thiffault, J. W. Langston, D. A. Cory-Slechta, and D. A. Di Monte, "Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat.," *Neurobiol. Dis.*, vol. 10, no. 2, pp. 119–27, Jul. 2002.

[371] B. K. Barlow, M. J. Thiruchelvam, L. Bennice, D. A. Cory-Slechta, N. Ballatori, and E. K. Richfield, "Increased synaptosomal dopamine content and brain concentration of paraquat produced by selective dithiocarbamates," *J. Neurochem.*, vol. 85, no. 4, pp. 1075–1086, 2003.

[372] A. H. Miller, V. Maletic, and C. L. Raison, "Inflammation and Its Discontents: The Role of Cytokines in the Pathophysiology of Major Depression," *Biological Psychiatry*, vol. 65, no. 9. pp. 732–741, 2009.

[373] V. Vasilevko, G. F. Passos, D. Quiring, E. Head, R. C. Kim, M. Fisher, and D. H. Cribbs, "Aging and cerebrovascular dysfunction: contribution of hypertension, cerebral amyloid angiopathy, and immunotherapy.," *Ann. N. Y. Acad. Sci.*, vol. 1207, pp. 58–70, Oct. 2010.

[374] Y. Pan, "Mitochondria, reactive oxygen species, and chronological aging: a message from yeast.," *Exp. Gerontol.*, vol. 46, no. 11, pp. 847–52, Nov. 2011.

[375] N. C. Derecki, N. Katzmarski, J. Kipnis, and M. Meyer-Luehmann, "Microglia as a critical player in both developmental and late-life CNS pathologies.," *Acta Neuropathol.*, vol. 128, no. 3, pp. 333–45, Sep. 2014.

[376] B. P. Lucke-Wold, A. F. Logsdon, R. C. Turner, C. L. Rosen, and J. D. Huber, "Aging, the metabolic syndrome, and ischemic stroke: redefining the approach for studying the blood-brain barrier in a complex neurological disease.," *Adv. Pharmacol.*, vol. 71, pp. 411–49, Jan. 2014.

[377] C. W. Blau, T. R. Cowley, J. O'Sullivan, B. Grehan, T. C. Browne, L. Kelly, A. Birch, N. Murphy, A. M. Kelly, C. M. Kerskens, and M. A. Lynch, "The age-related deficit in LTP is associated with changes in perfusion and blood-brain barrier permeability.," *Neurobiol. Aging*, vol. 33, no. 5, p. 1005.e23-35, May 2012.

[378] T. R. Chapman, R. M. Barrientos, J. T. Ahrendsen, J. M. Hoover, S. F. Maier, and S. L. Patterson, "Aging and infection reduce expression of specific brain-derived neurotrophic factor mRNAs in hippocampus.," *Neurobiol. Aging*, vol. 33, no. 4, p. 832.e1-14, Apr. 2012.

[379] M. Thiruchelvam, A. McCormack, E. K. Richfield, R. B. Baggs, A. W. Tank, D. A. Di Monte, and D. A. Cory-Slechta, "Age-related irreversible progressive nigrostriatal dopaminergic neurotoxicity in the paraquat and maneb model of the Parkinson's disease phenotype," *Eur. J. Neurosci.*, vol. 18, no. 3, pp. 589–600, Aug. 2003.

[380] D. A. Cook, G. T. Kannarkat, A. F. Cintron, L. M. Butkovich, K. B. Fraser, J. Chang, N. Grigoryan, S. A. Factor, A. B. West, J. M. Boss, and M. G. Tansey, "LRRK2 levels in immune cells are increased in Parkinson's disease," *npj Park. Dis.*, vol. 3, no. 1, p. 11, 2017.

[381] S. Hayley, S. Lacosta, Z. Merali, N. Van Rooijen, and H. Anisman, "Central monoamine and plasma corticosterone changes induced by a bacterial endotoxin: Sensitization and cross-sensitization effects," *Eur. J. Neurosci.*, vol. 13, no. 6, pp. 1155–1165, 2001.

[382] Z. Cao, Q. Yao, and S. Zhang, "MiR-146a activates WAVE2 expression and enhances phagocytosis in lipopolysaccharide-stimulated RAW264.7 macrophages," *American Journal of Translational Research*, vol. 7, no. 8. pp. 1467–1474, 2015.

[383] Y. Wolf, S. Yona, K.-W. Kim, and S. Jung, "Microglia, seen from the CX3CR1 angle," *Front. Cell. Neurosci.*, 2013.

[384] A. Reeve, E. Simcox, and D. Turnbull, "Ageing and Parkinson's disease: Why is advancing age the biggest risk factor?," *Ageing Research Reviews*, vol. 14, no. 1. pp. 19–30, 2014.

[385] H. F. Liu, P. W. L. Ho, G. C. T. Leung, C. S. C. Lam, S. Y. Y. Pang, L. Li, M. H. W. Kung, D. B. Ramsden, and S. L. Ho, "Combined LRRK2 mutation, aging and chronic low dose oral rotenone as a model of Parkinson's disease," *Sci. Rep.*, vol. 7, 2017.

[386] N. Joseph, G. Biber, S. Fried, B. Reicher, O. Levy, B. Sabag, E. Noy, and M. Barda-Saad, "A conformational change within the WAVE2 complex regulates its degradation following cellular activation," *Scientific Reports*, vol. 7. 2017.

[387] K. Kawamura, K. Takano, S. Suetsugu, S. Kurisu, D. Yamazaki, H. Miki, T. Takenawa, and T. Endo, "N-WASP and WAVE2 acting downstream of phosphatidylinositol 3-kinase are required for myogenic cell migration induced by hepatocyte growth factor.," *J. Biol. Chem.*, vol. 279, no. 52, pp. 54862–54871, Dec. 2004.

[388] A. A. Staniland, A. K. Clark, R. Wodarski, O. Sasso, F. Maione, F. D'Acquisto, and M. Malcangio, "Reduced inflammatory and neuropathic pain and decreased spinal microglial response in fractalkine receptor (CX3CR1) knockout mice," *J. Neurochem.*, vol. 114, no. 4, pp. 1143–1157, 2010.

[389] H.-J. Kim, A. B. DiBernardo, J. A. Sloane, M. N. Rasband, D. Solomon, B. Kosaras, S. P. Kwak, and T. K. Vartanian, "WAVE1 Is Required for Oligodendrocyte Morphogenesis and Normal CNS Myelination," *J. Neurosci.*, vol. 26, no. 21, p. 5849 LP-5859, May 2006.

[390] P. M. Hughes, M. S. Botham, S. Frentzel, A. Mir, and V. H. Perry, "Expression of fractalkine (CX3CL1) and its receptor, CX3CR1, during acute and chronic inflammation in the rodent CNS.," *Glia*, vol. 37, no. 4, pp. 314–327, Mar. 2002.

[391] J. K. Harrison, Y. Jiang, S. Chen, Y. Xia, D. Maciejewski, R. K. McNamara, W. J. Streit, M. N. Salafranca, S. Adhikari, D. A. Thompson, P. Botti, K. B. Bacon, and L. Feng, "Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia," *Proc. Natl. Acad. Sci.*, vol. 95, no. 18, pp. 10896–10901, 1998.

[392] D. Maciejewski-Lenoir, S. Chen, L. Feng, R. Maki, and K. B. Bacon, "Characterization

of fractalkine in rat brain cells: migratory and activation signals for CX3CR-1-expressing microglia.," *J. Immunol.*, vol. 163, no. 3, pp. 1628–35, 1999.

[393] D. Bérangère Ré and S. Przedborski, "Fractalkine: Moving from chemotaxis to neuroprotection," *Nature Neuroscience*, vol. 9, no. 7. pp. 859–861, 2006.

[394] V. Zujovic, J. Benavides, X. Vigé, C. Carter, and V. Taupin, "Fractalkine modulates TNF-α secretion and neurotoxicity induced by microglial activation," *Glia*, vol. 29, no. 4, pp. 305–315, 2000.

[395] X.-Y. Qin, S.-P. Zhang, C. Cao, Y. P. Loh, and Y. Cheng, "Aberrations in Peripheral Inflammatory Cytokine Levels in Parkinson Disease: A Systematic Review and Meta-analysis.," *JAMA Neurol.*, vol. 73, no. 11, pp. 1316–1324, 2016.

[396] N. Dzamko, D. B. Rowe, and G. M. Halliday, "Increased peripheral inflammation in asymptomatic leucine-rich repeat kinase 2 mutation carriers," *Mov. Disord.*, vol. 31, no. 6, pp. 889–897, Jun. 2016.

[397] B. Macchi, D. R. Paola, F. Marino-Merlo, M. R. Felice, S. Cuzzocrea, and A. Mastino, "Inflammatory and Cell Death Pathways in Brain and Peripheral Blood in Parkinson's disease," *CNS Neurol. Disord. - Drug Targets*, vol. 14, no. 3, pp. 313–324, 2015.

[398] M. C. Hernández-Romero, M. J. Delgado-Cortés, M. Sarmiento, R. M. de Pablos, A. M. Espinosa-Oliva, S. Argüelles, M. J. Bández, R. F. Villarán, R. Mauriño, M. Santiago, J. L. Venero, A. J. Herrera, J. Cano, and A. Machado, "Peripheral inflammation increases the deleterious effect of CNS inflammation on the nigrostriatal dopaminergic system," *Neurotoxicology*, vol. 33, no. 3, pp. 347–360, 2012.

[399] A. Machado, A. J. Herrera, J. L. Venero, M. Santiago, R. M. De Pablos, R. F. Villarán, A. M. Espinosa-Oliva, S. Argüelles, M. Sarmiento, M. J. Delgado-Cortés, R. Mauriño, and J. Cano, "Peripheral Inflammation Increases the Damage in Animal Models of Nigrostriatal Dopaminergic Neurodegeneration: Possible Implication in Parkinson's Disease Incidence," *Parkinsons. Dis.*, vol. 2011, pp. 1–10, 2011.

[400] S. Wang, X. Zhu, L. Xiong, and J. Ren, "Ablation of Akt2 prevents paraquat-induced myocardial mitochondrial injury and contractile dysfunction: Role of Nrf2," *Toxicol.*

Lett., vol. 269, pp. 1–14, 2017.

[401] B. Riahi, H. Rafatpanah, M. Mahmoudi, B. Memar, A. Fakhr, N. Tabasi, and G. Karimi, "Evaluation of suppressive effects of paraquat on innate immunity in Balb/c mice," *J. Immunotoxicol.*, vol. 8, no. 1, pp. 39–45, Mar. 2011.

[402] B. Riahi, H. Rafatpanah, M. Mahmoudi, B. Memar, A. Brook, N. Tabasi, and G. Karimi, "Immunotoxicity of paraquat after subacute exposure to mice," *Food Chem. Toxicol.*, vol. 48, no. 6, pp. 1627–1631, 2010.

[403] D. Litteljohn and S. Hayley, "Cytokines as potential biomarkers for Parkinson's disease: A multiplex approach," *Methods in Molecular Biology*, vol. 934. pp. 121–144, 2012.

[404] S. L. Rodriguez-Zas, S. E. Nixon, M. A. Lawson, R. H. Mccusker, B. R. Southey, J. C. O'Connor, R. Dantzer, and K. W. Kelley, "Advancing the understanding of behaviors associated with Bacille Calmette Guerin infection using multivariate analysis," *Brain. Behav. Immun.*, vol. 44, pp. 176–186, 2015.

[405] G. Van Maele-Fabry, P. Hoet, F. Vilain, and D. Lison, "Occupational exposure to pesticides and Parkinson's disease: A systematic review and meta-analysis of cohort studies," *Environment International*. 2012.

[406] D. A. Cook, G. T. Kannarkat, A. F. Cintron, L. M. Butkovich, K. B. Fraser, J. Chang, N. Grigoryan, S. A. Factor, A. B. West, J. M. Boss, and M. G. Tansey, "LRRK2 levels in immune cells are increased in Parkinson's disease," *npj Park. Dis.*, vol. 3, no. 1, p. 11, 2017.

[407] R. Fu, Q. Shen, P. Xu, J. J. Luo, and Y. Tang, "Phagocytosis of microglia in the central nervous system diseases," *Mol. Neurobiol.*, 2014.

[408] A. Meixner, K. Boldt, M. Van Troys, M. Askenazi, C. J. Gloeckner, M. Bauer, J. A. Marto, C. Ampe, N. Kinkl, and M. Ueffing, "A QUICK Screen for Lrrk2 Interaction Partners – Leucine-rich Repeat Kinase 2 is Involved in Actin Cytoskeleton Dynamics," *Mol. Cell. Proteomics*, 2011.

[409] K. Kierdorf and M. Prinz, "Factors regulating microglia activation," *Front. Cell.*

Neurosci., 2013.

[410] R. C. Paolicelli, K. Bisht, and M.-Ã. Tremblay, “Fractalkine regulation of microglial physiology and consequences on the brain and behavior,” *Front. Cell. Neurosci.*, 2014.

[411] T. Mizuno, J. Kawanokuchi, K. Numata, and A. Suzumura, “Production and neuroprotective functions of fractalkine in the central nervous system,” *Brain Res.*, vol. 979, no. 1–2, pp. 65–70, 2003.

[412] M. M. Pabon, A. D. Bachstetter, C. E. Hudson, C. Gemma, and P. C. Bickford, “CX3CL1 reduces neurotoxicity and microglial activation in a rat model of Parkinson’s disease.,” *J. Neuroinflammation*, vol. 8, p. 9, Jan. 2011.

[413] K. R. Nash, P. Moran, D. J. Finneran, C. Hudson, J. Robinson, D. Morgan, and P. C. Bickford, “Fractalkine over expression suppresses α-synuclein-mediated neurodegeneration,” *Mol. Ther.*, 2015.

[414] B. Ma, L. Xu, X. Pan, L. Sun, J. Ding, C. Xie, V. E. Koliatsos, and H. Cai, “LRRK2 modulates microglial activity through regulation of chemokine (C–X3–C) receptor 1 – mediated signalling pathways,” *Hum. Mol. Genet.* , Jul. 2016.

[415] J. Barlic, D. H. McDermott, M. N. Merrell, J. Gonzales, L. E. Via, and P. M. Murphy, “Interleukin (IL)-15 and IL-2 Reciprocally Regulate Expression of the Chemokine Receptor CX3CR1 through Selective NFAT1- and NFAT2-dependent Mechanisms,” *J. Biol. Chem.* , vol. 279, no. 47, pp. 48520–48534, Nov. 2004.

[416] B. Lu, “LRRK2 directing ER-to-Golgi transport? (ER)yES!,” *EMBO J.*, vol. 33, no. 20, pp. 2279–2280, Oct. 2014.

[417] M. Lichtenberg, A. Mansilla, V. R. Zecchini, A. Fleming, and D. C. Rubinsztein, “The Parkinson’s disease protein LRRK2 impairs proteasome substrate clearance without affecting proteasome catalytic activity,” *Cell Death Dis.*, 2011.

[418] J. Alegre-Abarrategui, H. Christian, M. M. P. Lufino, R. Mutihac, L. L. Venda, O. Ansorge, and R. Wade-Martins, “LRRK2 regulates autophagic activity and localizes to specific membrane microdomains in a novel human genomic reporter cellular model,” *Hum. Mol. Genet.*, 2009.

[419] T. Mizuno, G. Zhang, H. Takeuchi, J. Kawanokuchi, J. Wang, Y. Sonobe, S. Jin, N. Takada, Y. Komatsu, and A. Suzumura, "Interferon-gamma directly induces neurotoxicity through a neuron specific, calcium-permeable complex of IFN- gamma receptor and AMPA GluR1 receptor.," *FASEB J.*, 2008.

[420] B. Jabri and L. B. Barreiro, "Don't move: LRRK2 arrests NFAT in the cytoplasm," *Nature Immunology*. 2011.

[421] A. Drinkut, K. Tillack, D. P. Meka, J. B. Schulz, S. Kügler, and E. R. Kramer, "Ret is essential to mediate GDNF's neuroprotective and neuroregenerative effect in a Parkinson disease mouse model," *Cell Death Dis.*, 2016.

[422] B. J. Hoffer and B. K. Harvey, "Parkinson disease: Is GDNF beneficial in Parkinson disease?," *Nat. Rev. Neurol.*, 2011.

[423] L. Lorigados Pedre, N. Pavón Fuentes, L. Alvarez González, A. McRae, T. Serrano Sánchez, L. Blanco Lescano, and R. Macías González, "Nerve growth factor levels in parkinson disease and experimental parkinsonian rats," *Brain Res.*, 2002.

[424] M. E. Woodbury and T. Ikezu, "Fibroblast growth factor-2 signaling in neurogenesis and neurodegeneration," *Journal of Neuroimmune Pharmacology*. 2014.

[425] S. Gua, H. Huanga, J. Bia, Y. Yao, and T. Wen, "Combined treatment of neurotrophin-3 gene and neural stem cells is ameliorative to behavior recovery of Parkinson's disease rat model," *Brain Res.*, 2009.

[426] E.-H. Joe, D.-J. Choi, J. An, J.-H. Eun, I. Jou, and S. Park, "Astrocytes, Microglia, and Parkinson's Disease," *Exp. Neurobiol.*, vol. 27, no. 2, pp. 77–87, Apr. 2018.

[427] W. S. Wandu, C. Tan, O. Ogbeifun, B. P. Vistica, G. Shi, S. J. H. Hinshaw, C. Xie, X. Chen, D. M. Klinman, H. Cai, and I. G. Gery, "Leucine-rich repeat kinase 2 (Lrrk2) deficiency diminishes the development of experimental autoimmune uveitis (EAU) and the adaptive immune response," *PLoS One*, vol. 10, no. 6, 2015.

[428] J. Schapansky, J. D. Nardozzi, and M. J. LaVoie, "The complex relationships between microglia, alpha-synuclein, and LRRK2 in Parkinson's disease," *Neuroscience*, vol. 302, 2015.

[429] E. Janda, L. Boi, and A. R. Carta, “Microglial Phagocytosis and Its Regulation: A Therapeutic Target in Parkinson’s Disease?,” *Front. Mol. Neurosci.*, 2018.

[430] P. L. McGeer, S. Itagaki, B. E. Boyes, and E. G. McGeer, “Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson’s and Alzheimer’s disease brains.,” *Neurology*, vol. 38, no. 8, pp. 1285–1291, 1988.

[431] J. J. Neher, U. Neniskyte, J.-W. Zhao, A. Bal-Price, A. M. Tolkovsky, and G. C. Brown, “Inhibition of Microglial Phagocytosis Is Sufficient To Prevent Inflammatory Neuronal Death,” *J. Immunol.*, 2011.

[432] M. Fricker, M. J. Oliva-Martín, and G. C. Brown, “Primary phagocytosis of viable neurons by microglia activated with LPS or Aβ is dependent on calreticulin/LRP phagocytic signalling,” *J. Neuroinflammation*, vol. 9, p. 196, Aug. 2012.

[433] S. R. Mulay, J. Desai, S. V. Kumar, J. N. Eberhard, D. Thomasova, S. Romoli, M. Grigorescu, O. P. Kulkarni, B. Popper, V. Vielhauer, G. Zuchtriegel, C. Reichel, J. H. Bräsen, P. Romagnani, R. Bilyy, L. E. Munoz, M. Herrmann, H. Liapis, S. Krautwald, A. Linkermann, and H.-J. Anders, “Eaten alive! Cell death by primary phagocytosis: ‘phagoptosis,’” *Nat. Commun.*, 2016.

[434] J. V. Emmrich, T. C. Hornik, J. J. Neher, and G. C. Brown, “Rotenone induces neuronal death by microglial phagocytosis of neurons,” in *FEBS Journal*, 2013.

[435] U. Neniskyte, J. J. Neher, and G. C. Brown, “Neuronal death induced by nanomolar amyloid beta is mediated by primary phagocytosis of neurons by microglia,” *J. Biol. Chem.*, 2011.

[436] H. Y. Heo, J. M. Park, C. H. Kim, B. S. Han, K. S. Kim, and W. Seol, “LRRK2 enhances oxidative stress-induced neurotoxicity via its kinase activity,” *Exp. Cell Res.*, 2010.

[437] J. Q. Li, L. Tan, and J. T. Yu, “The role of the LRRK2 gene in Parkinsonism,” *Molecular neurodegeneration*. 2014.

[438] M. Westerlund, A. C. Belin, A. Anvret, P. Bickford, L. Olson, and D. Galter, “Developmental regulation of leucine-rich repeat kinase 1 and 2 expression in the brain

and other rodent and human organs: Implications for Parkinson's disease," *Neuroscience*, vol. 152, no. 2, pp. 429–436, Mar. 2008.

[439] M. A. S. Baptista, K. D. Dave, M. A. Frasier, T. B. Sherer, M. Greeley, M. J. Beck, J. S. Varsho, G. A. Parker, C. Moore, M. J. Churchill, C. K. Meshul, and B. K. Fiske, "Loss of leucine-rich repeat kinase 2 (LRRK2) in rats leads to progressive abnormal phenotypes in peripheral organs," *PLoS One*, vol. 8, no. 11, 2013.

[440] E. Kozina, S. Sadasivan, Y. Jiao, Y. Dou, Z. Ma, H. Tan, K. Kodali, T. Shaw, J. Peng, and R. J. Smeyne, "Mutant LRRK2 mediates peripheral and central immune responses leading to neurodegeneration in vivo," *Brain*, pp. awy077-awy077, Mar. 2018.

[441] M. Kuss, E. Adamopoulou, and P. J. Kahle, "Interferon-γ induces leucine-rich repeat kinase LRRK2 via extracellular signal-regulated kinase ERK5 in macrophages," *J. Neurochem.*, 2014.

[442] W. A. Banks and M. A. Erickson, "The blood-brain barrier and immune function and dysfunction," *Neurobiology of Disease*, vol. 37, no. 1. pp. 26–32, 2010.

[443] A. Louveau, I. Smirnov, T. J. Keyes, J. D. Eccles, S. J. Rouhani, J. D. Peske, N. C. Derecki, D. Castle, J. W. Mandell, K. S. Lee, T. H. Harris, and J. Kipnis, "Structural and functional features of central nervous system lymphatic vessels," *Nature*, vol. 523, no. 7560, pp. 337–341, 2015.

[444] W. Pan, K. P. Stone, H. Hsuchou, V. K. Manda, Y. Zhang, and A. J. Kastin, "Cytokine Signaling Modulates Blood-Brain Barrier Function," *Curr. Pharm. Des.*, vol. 17, no. 33, p. 3729, 2011.

[445] Q. Chai, R. She, Y. Huang, and Z. F. Fu, "Expression of Neuronal CXCL10 Induced by Rabies Virus Infection Initiates Infiltration of Inflammatory Cells, Production of Chemokines and Cytokines, and Enhancement of Blood-Brain Barrier Permeability," *Journal of Virology*, vol. 89, no. 1. 1752 N St., N.W., Washington, DC, pp. 870–876, Jan-2015.

[446] A. M. S. Hartz, B. Bauer, G. Fricker, and D. S. Miller, "Rapid modulation of P-glycoprotein-mediated transport at the blood-brain barrier by tumor necrosis factor-

alpha and lipopolysaccharide.," *Mol. Pharmacol.*, vol. 69, no. 2, pp. 462–70, 2006.

[447] B. Bauer, A. M. S. Hartz, and D. S. Miller, "Tumor necrosis factor alpha and endothelin-1 increase P-glycoprotein expression and transport activity at the blood-brain barrier.," *Mol. Pharmacol.*, vol. 71, no. 3, pp. 667–75, 2007.

[448] R. H. McCusker and K. W. Kelley, "Immune-neural connections: how the immune system's response to infectious agents influences behavior," *J Exp Biol*, vol. 216, no. Pt 1, pp. 84–98, 2013.

[449] C. H. Williams-Gray, R. Wijeyekoon, A. J. Yarnall, R. A. Lawson, D. P. Breen, J. R. Evans, G. A. Cummins, G. W. Duncan, T. K. Khoo, D. J. Burn, and R. A. Barker, "Serum immune markers and disease progression in an incident Parkinson's disease cohort (ICICLE-PD).," *Mov. Disord.*, vol. 31, no. 7, pp. 995–1003, Jul. 2016.

[450] K. Brockmann, A. Apel, C. Schulte, N. Schneiderhan-Marra, C. Pont-Sunyer, D. Vilas, J. Ruiz-Martinez, M. Langkamp, J.-C. Corvol, F. Cormier, T. Knorpp, T. O. Joos, T. Gasser, B. Schüle, J. O. Aasly, T. Foroud, J. F. Marti-Masso, A. Brice, E. Tolosa, C. Marras, D. Berg, and W. Maetzler, "Inflammatory Profile in LRRK2-associated prodromal and clinical PD," *Revis.*, vol. 13, no. 1, p. 122, May 2016.

[451] C. C. Ferrari and R. Tarelli, "Parkinson's disease and systemic inflammation.," *Parkinsons. Dis.*, vol. 2011, p. 436813, 2011.

[452] D. K. Stone, A. D. Reynolds, R. L. Mosley, and H. E. Gendelman, "Innate and adaptive immunity for the pathobiology of Parkinson's disease.," *Antioxid. Redox Signal.*, vol. 11, no. 9, pp. 2151–66, 2009.

[453] K. E. Olson and H. E. Gendelman, "Immunomodulation as a neuroprotective and therapeutic strategy for Parkinson's disease," *Current Opinion in Pharmacology*, vol. 26. pp. 87–95, 2016.

[454] C. Raposo, N. Graubardt, M. Cohen, C. Eitan, A. London, T. Berkutzki, and M. Schwartz, "CNS Repair Requires Both Effector and Regulatory T Cells with Distinct Temporal and Spatial Profiles," *J. Neurosci.*, 2014.

[455] J.-M. Taymans and E. Greggio, "LRRK2 Kinase Inhibition as a Therapeutic Strategy

for Parkinson's Disease, Where Do We Stand?," *Curr. Neuropharmacol.*, 2016.

[456] A. B. West, "Achieving neuroprotection with LRRK2 kinase inhibitors in Parkinson disease," *Experimental Neurology*. 2017.

[457] R. Boddu, T. D. Hull, S. Bolisetty, X. Hu, M. S. Moehle, J. P. L. Daher, A. I. Kamal, R. Joseph, J. F. George, A. Agarwal, L. M. Curtis, and A. B. West, "Leucine-rich repeat kinase 2 deficiency is protective in rhabdomyolysis-induced kidney injury," *Hum. Mol. Genet.*, 2015.

[458] E. Andres-Mateos, R. Mejias, M. Sasaki, X. Li, B. M. Lin, S. Biskup, L. Zhang, R. Banerjee, B. Thomas, L. Yang, G. Liu, M. F. Beal, D. L. Huso, T. M. Dawson, and V. L. Dawson, "Unexpected Lack of Hypersensitivity in LRRK2 Knock-Out Mice to MPTP (1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine)," *J. Neurosci.*, vol. 29, no. 50, pp. 15846–15850, 2009.

[459] M. Volta, S. Cataldi, D. Beccano-Kelly, L. Munsie, I. Tatarnikov, P. Chou, S. Bergeron, E. Mitchell, R. Lim, J. Khinda, A. Lloret, C. F. Bennett, C. Paradiso, M. Morari, M. J. Farrer, and A. J. Milnerwood, "Chronic and acute LRRK2 silencing has no long-term behavioral effects, whereas wild-type and mutant LRRK2 overexpression induce motor and cognitive deficits and altered regulation of dopamine release," *Park. Relat. Disord.*, 2015.

[460] D. A. Beccano-Kelly, N. Kuhlmann, I. Tatarnikov, M. Volta, L. N. Munsie, P. Chou, L.-P. Cao, H. Han, L. Tapia, M. J. Farrer, and A. J. Milnerwood, "Synaptic function is modulated by LRRK2 and glutamate release is increased in cortical neurons of G2019S LRRK2 knock-in mice," *Front. Cell. Neurosci.*, 2014.

[461] R. N. Fuji, M. Flagella, M. Baca, M. A. S. Baptista, J. Brodbeck, B. K. Chan, B. K. Fiske, L. Honigberg, A. M. Jubb, P. Katavolos, D. W. Lee, S. C. Lewin-Koh, T. Lin, X. Liu, S. Liu, J. P. Lyssikatos, J. O'Mahony, M. Reichelt, M. Roose-Girma, Z. Sheng, T. Sherer, A. Smith, M. Solon, Z. K. Sweeney, J. Tarrant, A. Urkowitz, S. Warming, M. Yaylaoglu, S. Zhang, H. Zhu, A. A. Estrada, and R. J. Watts, "Effect of selective LRRK2 kinase inhibition on nonhuman primate lung," *Sci. Transl. Med.*, 2015.

[462] D. A. Roosen and M. R. Cookson, “LRRK2 at the interface of autophagosomes, endosomes and lysosomes,” *Molecular Neurodegeneration*. 2016.